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Advances in Nanomaterials-based Cell Biology Research

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Chapter 1
Nanomaterials and Stem Cells for Bone Tissue Engineering

Tianxu Zhang and Ronghui Zhou

Abstract With the rapid development of nanotechnology, nanomaterials have been widely applied to bone regeneration. Stem cells, scaffold, and growth factors are commonly regarded as three crucial factors contributing to successful bone tissue engineering. The application of nanomaterials significantly improves the physico-chemical and biological properties of the scaffold, which could create biomimetic environment for the osteogenic differentiation of stem cells and sustained release of the growth factors. In this part, we focus on the discussion about the stem cells, nanomaterials, and growth factors which are applied in bone tissue engineering.

Keywords Nanomaterials · Stem cells · Bone regeneration · Tissue engineering

1.1 Introduction

Bone tissue is the most important supportive tissue which could continuously remodel and rebuild throughout the lifetime. Bone defects or bone fracture is common diseases affecting the normal function of skeletal system. Although there are internal self-repair and remodel for the pathological injuries, severe bone defect caused by traumas, tumor, or infection still need extra medical intervention. Bone grafts are also alternative candidates for the treatment of bone defects, but the sources are also limited and autogenous bone grafts could be invasive. Currently, nanomaterials have been applied to bone tissue engineering because of their unique nanoscale properties such as specific surface area, porosity, and mechanical property [1–3]. Seed cells, scaffold, and growth factors are considered to be three crucial factors for tissue engineering [4]. As an important part, the 3D scaffold plays a vital role in bone regeneration. A suitable scaffold can mimic the microenvironment of cell growth and provide biomimetic structures with good biocompatibility for cell

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proliferation and new bone growth [5, 6]. Due to the good biological property, nano-biomaterial has become an ideal material for the development of 3D scaffold for bone regeneration. Various nano-biomaterials such as nanocomposite materials, nanofiber materials, nano-bioactive materials, and injectable nanomaterials have been synthesized and used in the research of bone tissue engineering, presenting broad application prospects [7, 8].

Natural bone tissue consists of apatite and polymer collagen fibers, which have hierarchical structures and excellent mechanical properties. In order to mimic the biological structures, various nanomaterials and scaffolds are supposed to be applied to create biomimetic environment for stem cell osteogenic differentiation and bone regeneration [9]. The interaction between the stem cells and nanomaterials is extremely complex, which could be affected by many factors. The physicochemical and mechanical properties of different nanomaterials directly determined the biological potentials for bone regeneration. Understanding different properties of these nanomaterials is crucial for better regeneration results. How to perfectly combine different nanomaterials with complementary properties and precisely manipulate the osteogenesis differentiation of stem cell play key roles in current researches.

1.2 Stem Cell Types Applied to Nanomaterial-Based Bone Regeneration

Cells, scaffold, and growth factors are three crucial factors for tissue engineering. As special cells with multilineage differentiation capacity, stem cells are crucial for tissue engineering, which has revolutionized tissues engineering area, especially for the bone regeneration. Nowadays, many kinds of stem cells have been identified, which could be generally concluded into two different types: embryonic stem cell (ESC) and adult stem cell. They have been widely studied for tissue engineering because of their self-renewal capacity and multilineage differentiation potentials. But the application of ESCs is limited by their limited sources and ethical requirement. Adult stem cells are more commonly studied and applied to bone tissue engineering, and we will concentrate on the discussion about adult stem cells.

1.2.1 Mesenchymal Stem Cells (MSCs)

MSCs are the most common stem cell types which have multilineage differentiation and self-renewal capacity. As a multipotent stem cell, MSC could transform to osteoblast, chondrocyte, and adipocyte (Fig. 1.1). MSCs are firstly identified in the bone marrow, and then many other tissues were proved the existence of MSCs such as the skin, dental pulps, blood vessel, and adipose tissues. Since the first isolation in the 1950s, MSCs have been proved with multipotent and self-renewable capacities, which could differentiate into bone, muscles, adipose tissue, cartilage, and neural cell.

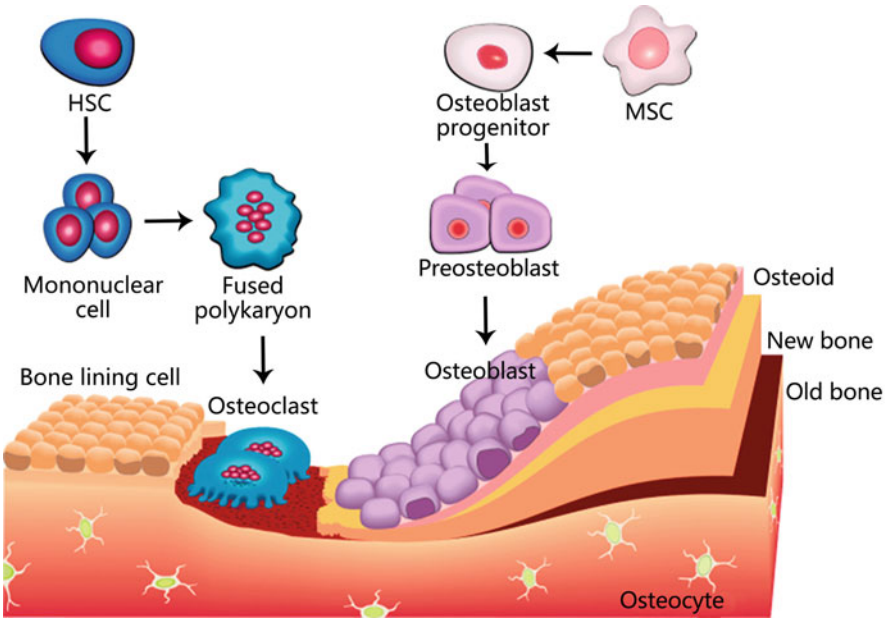

Fig. 1.1 The evolution process of osteoblasts and osteoclasts during the bone formation process. Reprinted with permission from ref. [10] Copyright (2015) Nature Publishing Group

1.2.1.1 Bone Marrow Stromal Cells (BMSCs)

Since they were firstly isolated in the 1960s, BMSCs have been widely applied to tissue engineering especially for bone tissue regeneration. Early in 1997, Komori et al. found BMSCs could express runt-related transcription factor 2 (Runx2), which was regarded as the key osteogenesis transcription factor [11]. In other words, BMSCs still retain the plasticity and stemness for potential osteogenesis differentiation [12–14].

BMSCs have been extensively studied for their osteogenic potentials since their isolation and identification. It is the earliest heterogeneous and primitive cell type found in the bone marrow, which are currently the most extensively applied cells for the bone tissue reconstruction and regeneration as a result of the easy obtain, culture, low immunogenicity, and easy transfection [15]. The identification of BMSCs is usually performed by flow cytometry and immunofluorescence staining. BMSCs mainly express surface markers such as CD29, CD44, and CD90 and don't express hematopoietic cell surface markers CD34 and CD45, which is the main difference in comparison with hematopoietic cells [16]. BMSCs can differentiate into osteoblasts under certain conditions and contribute to the productions, secretions, and mineralization of bone-related matrix, thereby achieving bone regeneration. The in vitro osteogenesis differentiation of BMSC largely depends on the osteogenesis induction culture medium, which include dexamethasone, β-glycerol phosphate, and vitamin

C. Dexamethasone enhances the osteogenesis of BMP-2 and stimulates RUNX2, ALP, OPN, and OCN expression; β-glycerol phosphate provides phosphorus ions and induces activation of ALP; vitamin C regulates the homeostasis of extracellular matrix collagen and promotes cell differentiation [17].

The combination treatment with BMSCs and biomaterials has been proved to be able to enhance the bone formation in vitro and in vivo. Many studies have proved that the local treatment of BMSC could accelerate the healing process of large-scale bone defect such as craniomaxillofacial defect. Although BMSCs have been widely applied to bone engineering and have great potentials for multilineage differentiation, limitations also exist. For example, the proliferation and differentiation abilities of BMSCs could possibly be declined after continuous culture and self-renewal ability could be limited. Moreover, the source from bone marrow is also limited. Furthermore, their differentiation potential could be also altered by different culture environment. More importantly, even if the BMSCs were purely isolated, only part of the BMSCs could be susceptible to osteogenesis [18]. In addition, the in vitro expansion of BMSCs could possibly cause immunological rejection responses after in vivo plantation.

1.2.1.2 Adipose-Derived Stem Cells (ASCs)

Besides BMSCs, the other abundant resource of mesenchymal stem cell is ASCs, which have also been applied to tissue engineering, especially for bone regeneration. Different from BMSCs, ASCs have advantages including easy access and isolation, less invasiveness, promising osteogenic ability, low immunogenicity, and immune regulation effects [19]. More importantly, ASCs are more abundant in sources by hundred folds [20]. ASCs were proved to have pluripotential ability of differentiating into other mesodermal lineages and ectodermal lineages. Despite the pluripotential ability, ADSCs lack the capacity to differentiate to the embryonic and extraembryonic tissue types like embryonic stem cells.

The surface markers expressed in ASCs include CD29, CD44, CD73, and CD90, but the hematopoietic-related surface markers CD34, CD45, and CD79 are negatively expressed, which are similar with the surface marker of BMSCs. Different from BMSCs, ASCs express CD36 and CD49d but do not express CD48f and CD104, which could be used to differentiate ASCs and BMSCs. In 2013, the International Fat Applied Technology Society defined the cell phenotype of ADSCs for uniform isolation and identification: (1) the phenotype of newly separated ADSCs is CD31 (−)/CD34 (+)/CD45 (−)/CD235a (−), and the phenotype of ADSCs cultured in vitro is CD31 (−)/CD44 (+)/CD45 (−)/CD73 (+)/CD90 (+)/CD105 (+) [21]. For biomedical application in bone regeneration, both the BMSC and ASC are very promising for osteogenic differentiation. However, the proliferation rate of ADSCs is faster than BMSCs. More importantly, ASCs could maintain their cellular activities in a good status including proliferation, differentiation, and metabolism under in vivo pathological environment [22]. Immunomodulatory effect is another specific characteristic of ADSCs, such as secretion of growth factors and

inflammatory factors, promoting angiogenesis and so on. ASCs maintained their anti-inflammatory ability and play important role for microenvironmental regulation in the pathological environment.

Generally, ASCs have similar characteristics for bone tissue engineering with BMSCs, but ASCs have several special capacities including more abundant in sources, faster proliferation, and immunoregulation capacity, which might promise better bone regeneration outcomes. But limitation and challenges also exist; the phenotypes of ASCs are different in vivo and in vitro. The phenotype will also change following continuous proliferation and differentiation such as CD34 expression. Furthermore, ASCs isolated from fat in different tissues may have discrepant differentiation potentials. Therefore, the mechanisms of induced differentiation require further investigation for better biomedical application of ASCs.

1.2.1.3 Dental Pulp Stem Cells (DPSCs)

As one important type of MSC, DPSCs are isolated from dental pulp tissue, which also have multiple differentiation capabilities. DPSCs were firstly isolated and identified by Gronthos et al. [23] in 2000. These MSC-like cells in dental pulp tissues also express the MSC markers like CD29, CD105, CD146, CD166, and STRO-1 [24]. DPSCs could be isolated in human third molar and exfoliated deciduous teeth (SHED). Miura et al. [25] firstly isolated DPSCs from SHED and applied them to in vivo bone tissue engineering. According to the different sources from permanent teeth and exfoliated deciduous teeth, there are some differences between the hDPSCs and SHED. SHEDs are isolated from deciduous teeth and they could be more immature than hDPSCs. In other words, SHEDs have stronger capacity in terms of proliferation and differentiation. Meanwhile, obtaining SHED from deciduous teeth could be easier, which is advantageous for clinical application [26]. Compared with DPSCs from normal teeth, DPSCs isolated from supernumerary teeth have higher proliferation capacity and differentiation potential [27].

Since the potential differentiation ability and accessibility, DPSCs also have potentials in bone tissue engineering. Dental pulp tissues are accessible organs and have recently attracted much attention for MSC isolation and tissue engineering. DPSCs have excellent proliferation capacity and could retain the characteristics of stem cells after cultured by many generations. Besides the multilineage differentiation, undifferentiated DPSCs also have immunoregulation capacity. DPSCs could suppress the proliferation of T cell and B cell, increase the number of regulatory T cell, and produce TGF-β, IL-6, IL-10, nitric oxide (NO), and prostaglandin(PG)-E2 [28].

Although dental pulp seems an alternative tissue for stem cell isolation, the use of DPSCs is also limited due to the small quantity and longer culture for enough cells for tissue engineering. Furthermore, the in vivo application for bone regeneration of DPSCs could be also limited. For example, in a histological analysis for 3-year transplant of DPSCs in human mandibles, the regenerated bone was compact bone and lack of vasculatures [29]. Therefore, the manipulation for the uncertain

differentiation still requires further study. Besides, biological activity of dental pulp tissue may be declined with the age increase, and the autologous sources of DPSCs could be limited. Meanwhile, it still needs long-term exploration about the immune rejection of allogeneic DPSCs after transplantation.

1.2.2 Other Types of Adult Stem Cells

Many tissues and organs have the capacity of repair and regeneration, in which many adult stem cells could be isolated and applied to regenerative medicine. These adult stem cells play their unique roles in regenerative medicine such as neural stem cells, periosteal stem cells, corneal stem cells, and so on.

1.3 Nanomaterials Applied to Stem Cell Osteogenic Differentiation

During the past decades, various types of nanomaterials have been exploited and applied to nanomedicine. Many nanomaterials have been proved to influence bio-response of stem cells like proliferation and differentiation. For bone regeneration, osteogenesis differentiation of stem cell is very crucial for new bone formation. Many researches have discussed the osteogenic effects of nanomaterials and their potentials for bone tissue engineering. Unique cellular responses could occur depending on different types of materials, which is summarized as follows.

1.3.1 Polymeric Nanomaterials

Polymeric NPs have been extensively introduced into biomedicine area because of the good biocompatibility and drug-loading capacity. Meanwhile, surface modification imparts polymeric NPs unlimited possibilities for better osteogenic induction capabilities. Besides, good biodegradability also contributes the extensive application of polymeric nanomaterials. For example, PLGA and chitosan are commonly used for tissue engineering. Chitosan is well known as a biocompatible, biodegradable, and nontoxic biomaterial, which has great potentials for physicochemical modifications due to its porosity, tensile strength, and biocompatibility [30]. For example, Wu et al. [31] fabricated chitosan NPs as carrier to deliver microRNA to MSCs, and enhanced delivery efficiency was observed. As a result, osteogenic differentiation of MSCs obviously increased. More importantly, chitosan NPs showed good biocompatibility and no toxicity to the MSCs. Similarly, Chen et al. [32] also used chitosan NPs as nano-carrier to deliver the stable modified hsa-miR-

199a-5p (agomir); this chitosan NPs/agomir complex significantly improved the in vivo bone regeneration. Besides drug carriers, polymeric nanomaterials could be employed as scaffolds for bone regeneration [33]. Generally speaking, polymeric nanomaterials could be used as promising candidate to regulate stem cell osteogenic differentiation and bone tissue regeneration.

1.3.2 Metal-Based Nanomaterials

As a common type of nanomaterials, metal-based nanomaterials also showed their potentials for bone regeneration. Due to the unique metallicity, metal-based nanomaterials could induce osteogenic differentiation by causing mechanical stress to the stem cells. Currently reported osteogenic metal-based nanomaterials include gold NPs (AuNPs), silver NPs (AgNPs), titanium NPs (TiNPs), and iron NPs (FeNPs), and their osteogenic potentials are discussed as follows.

AuNPs could be regarded as promising nanomaterial for tissue engineering because they have satisfying biocompatibility, easy modification, and antimicrobial ability [34]. Naturally, many studies have reported their potentials for bone regeneration as well. For example, Yi et al. [35] treated MSCs with AuNPs and studied the cellular responses. The results turned out to be that AuNPs induced MSC osteogenic differentiation toward osteoblast cell rather than adipocyte cell. The underlying mechanism was that AuNPs could interact with the cell membrane and cytoplasm, which caused mechanical stress to the MSCs and activated osteogenesis-related gene expressions. More than MSCs, AuNPs were also proved to have osteogenic induction effect for human periodontal ligament stem cells (hPDLSCs). Niu et al. [36] investigated the induction of AuNPs for the osteogenic differentiation of hPDLSCs and detected osteogenic transcriptional profile of hPDLSCs after treated with AuNPs; the analysis suggested that the expressions of ALP, osterix, collagen I, and RUNX2 were significantly enhanced, which was important for osteogenic differentiation. In addition to pure AuNPs, easy functionalization and modification contribute to more extensive application of gold nanomaterials. Modified AuNPs were reported to enhance osteogenic differentiations of stem cell in many studies [37, 38].

Besides AuNPs, AgNPs also contributed to regulate the fate of stem cells. AgNPs are well known for their antimicrobial/antiviral properties and are often integrated into bone grafts as antimicrobial agents. Although the antibacterial activity of nanoscale silver nanomaterials is widely confirmed, the osteogenic properties remain controversial. For different kinds of stem cells, the results might be different. For example, Qin et al. [39] suggested that AgNPs could induce urine-derived stem cells differentiated toward osteogenic profile when AgNPs were at proper concentrations (for instance, 4 ug/mL). However, when the seed cells came to hMSCs, the results might be different. Liu et al. [40] suggested that AgNPs caused cytotoxicity to hMSCs and AgNPs didn't change the osteogenesis-related gene expression, which meant that AgNPs didn't induce the osteogenic differentiation of hMSCs. Therefore,

the osteogenic properties may vary according to different circumstances and seed cells as well. Although the antimicrobial effects may be advantageous for the use of AgNPs in bone regeneration, the cytotoxicity is also a nonnegligible problem for AgNPs [41].

Other types of metal-based nanomaterials also have positive influence for their osteogenic properties, which makes them play unique roles in bone regeneration and tissue engineering such as TiO_2NPs [42], iron oxide NPs [43], and so on.

1.3.3 Silica-Based Nanomaterials

Silica is one of the important elements for skeletal system, and silica-based nanomaterials are promising biomaterials due to their good biocompatibility [44]. It was proved that silica NPs showed no negative effect to the cell viability and exhibit size- and dose-independent cytocompatibility on hMSCs [45]. Furthermore, the ALP activity and bone nodule production of hMSCs were obviously enhanced after treated by silica NPs, which demonstrated the osteogenic induction effect of silica NPs. The osteogenic effects may derive from the Si release from the silica NPs as a result of cellular lysosomal degradation. Besides biocompatibility, porousness is the other unique property for silica nanomaterials. Due to the chemical modification property, nanoporously structured silica NPs attracted much interests in bone tissue engineering. Chemical modification could enhance the osteoinductive effect of silica NPs. Christel et al. [46] modified the nanoporous silica materials with bone growth factor BMP-2; the complex showed obvious osteoinductive effects on ASCs. Same osteoinductive effects could be found in other studies with different modification and composites [47, 48].

1.3.4 Carbon-Based Nanomaterials

Carbon nanomaterials have drawn increasing interests in biomedical application because of the excellent physicochemical and biocompatible characteristics [49, 50]. Their uniquely manipulative spatial structures including 2D and 3D impart them more structural possibilities for scaffold fabrication in tissue engineering, which could simulate the structure of biological bone extracellular matrix. Graphene (GR), graphene oxide (GR), and carbon nanotube (CNT) are common forms of carbon-based nanomaterials, which could be applied to bone tissue engineering.

Since the first report in 2004 of graphene by Novoselov and Geim, GR has been extensively applied to biomedical area due to its extraordinary physicochemical properties [51]. As single-layer 2D nanosheets, many studies have reported their positive impacts on the stem cell regulation [52, 53]. GR could provide a biocompatible scaffold for hMSCs and promote the osteogenic differentiation [54]. CNT is a new type of nanomaterial which have special shape and morphology with a

cylindrical architecture, which make CNT a promising candidate for biomedicine [55]. Many studies have proved the osteoinductive effect of CNTs, and the array of CNTs could affect the stem cell responses. It was suggested that only single-walled CNT without any other chemical/biochemical treatment could initiate osteogenic differentiation of hMSCs [56]. If hMSCs were cultivated on the multiwalled carbon nanotube (MWCNT) arrays, the cells showed different behaviors like well-spread and spiral-shaped cell colons, and osteocalcin (OCN) gene expression was enhanced in comparison with hMSCs cultured on dish [57]. Moreover, the combination of GR and CNT could also serve as osteoinductive hybrids. Yan et al. [49] fabricated GR/SWCNT complex and treated rat MSCs with these hybrids. After treatment by GR/SWCNT complex, osteogenic-related gene expressions and mineralized matrix nodule formation were enhanced. On the contrary, adipocyte-related genes were downregulated.

1.3.5 Nucleic Acid-Based Nanomaterials

As a novel type of nanomaterial, nucleic acid nanomaterials have drawn rising attention due to their excellent biocompatibility and editability. Nucleic acids (DNA, RNA) and nucleic acid analogs such as PNA and LNA play important roles in regulating gene and protein expression, which finally manipulate cell activities such as proliferation, migration, and differentiation [58]. DNA nanomaterials are more widely studied due to their self-assemble property according to the principle of Watson-Crick base pairing. As a result, various types of DNA origami have been reported with unique spatial structure and biological activities.

Our previous work has studied one of the DNA origamis, tetrahedral framework DNA nanostructures (TFNAs). Due to their tetrahedral nanostructure, cellular uptake of TFNAs could be more efficient than oligonucleotides. The multiple biological effects of TFNAs were extensively investigated including promoting cell migrations, proliferations, and differentiations, which suggested the great potentials of TFNAs in the tissue engineering area [59]. Zhou et al. [60] proved that TFNAs could promote the proliferation and osteogenic/odontogenic differentiations of DPSCs as the osteogenic-related gene and protein expressions were enhanced. Shao et al. [61] studied the effects of TFNAs on the osteogenic differentiations of ADSCs and found that TFNAs activated osteogenic potential of ADSCs via Wnt/β-catenin signaling pathway. TFNAs could also serve as novel drug carriers for functional nucleic acids like siRNA, microRNA, lncRNA, and oligonucleotides to achieve better bone regeneration results.

1.3.6 Hydroxyapatite

As basic components of biological bone tissue, hydroxyapatite has been widely applied to bone regeneration because of the satisfying biocompatibility and bioactivity. Natural bone tissue has hierarchical structures which mainly composed of periodically arranged inorganic nano-hydroxyapatites and organic collagen fibers. HA-based bioceramics have excellent osteoinductive and osteoconductive activity; the microporous structure of the material could lead to the high adsorption and accumulation of various endogenous bone growth factors, which will activate the differentiation of MSCs into osteoblasts and ultimately achieve osteogenesis induction. But the mechanical properties of HA prepared by the existing process are not good enough, which limits its wider application. Therefore, nano-HA/polymer composite biomaterials are more commonly applied for better mechanical properties which we will discuss in other parts. Although there are many types of HA/polymer composites, the standard of the properties requires to be unified; long-term follow-up is required to evaluate the clinical potentials.

1.4 Properties of Nanomaterials Affecting Osteogenic Differentiation and Bone Formation

The osteogenic differentiation of stem cells and bone formation process have intimate connection with the chemical, physical, mechanical, and biological properties of related nanomaterials as shown in Fig. 1.2.

1.4.1 Mechanical Properties

Bone tissues have strong load-bearing ability which consists of HA nanocrystals and fiber-shaped collagen molecules. One of the goals for bone regeneration is to simulate the hierarchical structure of biological bone tissue. Optimal scaffolds are supposed to have similar mechanical property to the natural bone to provide biomimetic environment for osteogenic differentiation of stem cells. For severe bone defect area, scaffolds should provide structural support for the bone regeneration. The matrix stiffness also plays important roles in osteogenic differentiation of stem cells [62]. Therefore, mechanical property of scaffold materials is very crucial for successful bone regeneration results, and suitable mechanical property seems to be the most basic requirement for bone regeneration scaffold.

Stem cells are not only regulated by biological molecular signals such as growth factors but also regulated by mechanical properties of scaffolds [63]. The mechanical signals will induce cell differentiation to different subtypes. Polymers and bioceramics are common materials which could provide suitable mechanical and

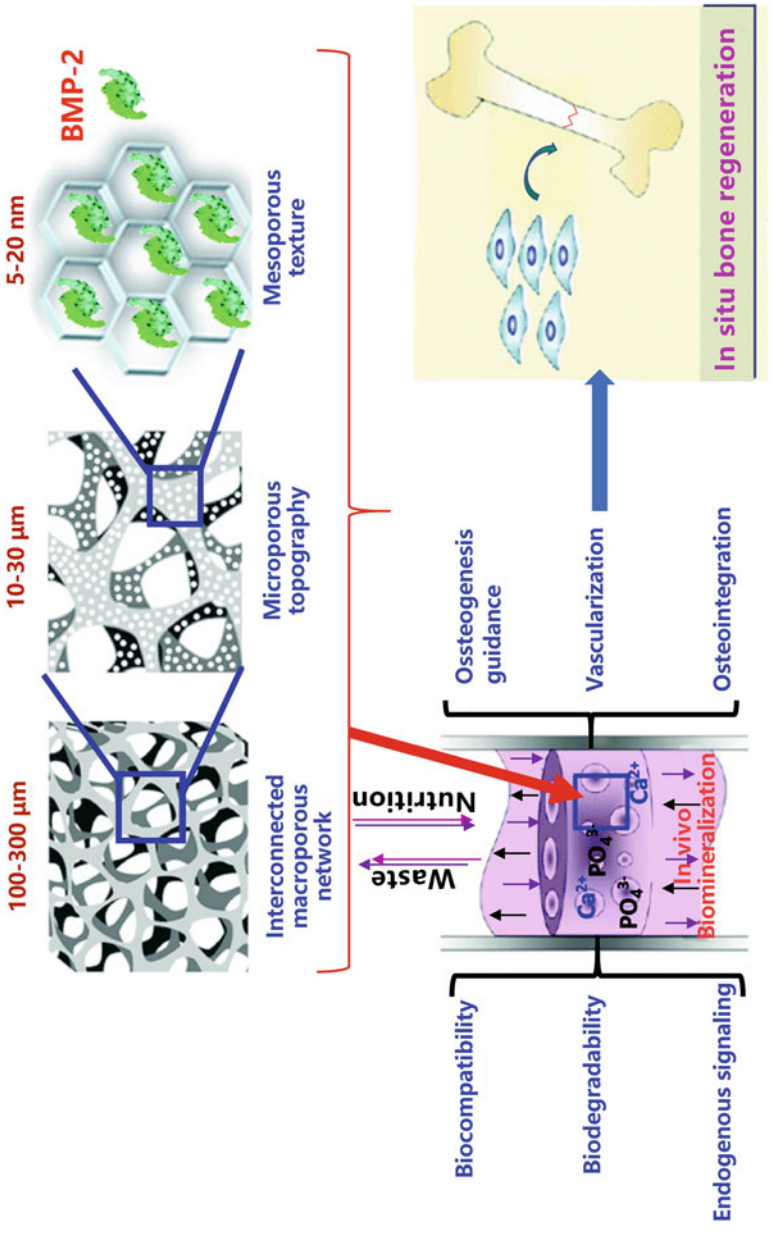

Fig. 1.2 The illustration of ideal properties and structures for nanomaterial-based bone regeneration. Reprinted with permission from ref. [1] Copyright (2017) The Royal Society of Chemistry

structural support for bone regeneration. Although polymers are reported to be useful in bone regeneration, single type of polymer seems not to satisfy the mechanical requirement. Therefore, the combination of polymers and inorganic materials is usually more common to improve the mechanical properties for better engineering. As essential component of natural bone tissues, HA is the most commonly used material to improve mechanical properties of the nanocomposite to better mimic the microstructures of biological bone [64]. Wei et al. [65] analyzed the structural effects of nano-HA/polymer composite scaffold for bone regeneration, they found that combination of nano-HA with the PLLA polymers enhanced the mechanical property by about two folds with suitable microarchitecture, which could be favorable for cell adherence and differentiation. Other types of materials could be also used to improve better mechanical property. Zhang et al. [66] incorporated octadecylamine-functionalized nanodiamond into PLLA polymers and studied the effect of mechanical properties changes on the bone formation process. The results demonstrated that incorporation of 10% wt nanodiamond obviously enhanced the tensile property of the composites. The increase in mechanical property increased the mineralization and bonelike apatite growth.

The hierarchical structures provide natural bone with excellent mechanical and biological properties. Therefore, the mechanical property of bone tissue scaffold should mimic the natural bone, which means that compression modulus should be 45–100 MPa. Different types of polymers and inorganic phases could be served to develop scaffolds with varied mechanical properties via adjusting ratio and conjugation manners of different components.

1.4.2 Porosity

Porosity is another crucial factor contributing to successful development of bone tissue scaffold. There are also requirements for void ratio and pore sizes to provide better environment for bone regeneration. Proper pore size and ratio are favorable for cell ingrowth and nutrition/waste exchanges. Too small pore size will prevent the cell ingrowth and may lead to cellular capsules around the scaffolds. Meanwhile, too large pore size could possibly reduce the surface area and mechanical strength of the scaffolds [9].

Murphy et al. [67] investigated the impact of pore size on the cellular adherence, proliferation, and migration on the porous scaffolds with 85–325 μm pore sizes. Although the final number of osteoblasts was the most abundant after 7-day observation for the biggest pore size, there was a suddenly increased peak for the 120 μm pore size scaffold. This might suggest that pore size was related to the surface area, which plays important roles in inducing initial cell attachment, because scaffold with large pore size has smaller surface area. The results also suggested that the cell adherence could not always be explained by surface area; if the range of pore sizes was 85–325 um, the surface area theory couldn't explain. After the cell attachment,

bigger pore size could provide more space for the cell proliferation and migration; finally they suggested that 325 um pore size was suitable for bone tissue engineering.

Since decades ago, discussion about the impact of pore sizes on the bone regeneration has been emerging. Pore sizes from tens to thousands microns have been reported for bone regeneration. An early study suggested that the ideal pore size for optimal bone in-growth rates was 100–135 um [68]. There were also studies suggesting that bone formation and vascularization required the pore size bigger than 300 μm. If the pore sizes are <300 μm, the scaffold tended to induce osteochondral ossification rather than osteogenesis [69, 70]. However, there were also study investigating the osteoinductive ability of nanoporous titanium with pore size of 30 nm and 100 nm; the results demonstrated that only substrates with 30 nm pores induced osteogenic differentiation of human neural crest-derived stem cells and substrates with 100 nm pore size didn't induce osteogenic differentiation [71]. There are evidences suggesting that macropores (>100 μm) are favorable for bone ingrowth and angiopoiesis, but microporosity (pore size <20 um) is also regarded as important way to improve the osteoinductive ability of scaffold. Microporosity could provide the scaffold with larger surface area and better permeability, which could enhance protein adsorption on the scaffold and improve cell-scaffold interaction [72]. Besides surface area, micropore-induced capillarity could also enhance the cell adherence, bone growth, and distribution in the scaffold [73, 74]. Besides the effect of pore size, porosity ratio is also a crucial factor for the bone formation and mechanical property of scaffold. Chen et al. [75] developed porous titanium scaffold for bone regeneration; the 30–50% porosity samples were similar with the structure of natural bone. hMSCs easily adhered and proliferated on the surface and grow into the porosity structures also indicated osseointegration potentials.

Porosity contributes to the regulation of bone tissue ingrowth and is an essential factor for successful bone regeneration results. Adequate pore size contributed to high surface area, osteogenic protein adsorption, and cell adherence and ingrowth. It has been preferably considered that if the pore size is between 90 and 200 um, it could induce better bone formation. But for different materials and stem cells, the porosity could be different for the optimal osteoinductive outcomes.

1.4.3 Hydrophilicity

The hydrophilicity of the material surface is an important factor affecting the cell behaviors like adhesion and morphology. The hydrophilicity decrease of scaffold could lead to poor cell adherence [76]. There are many factors that affect the hydrophilicity of the material such as surface roughness, surface topology, and surface physicochemical conditions, which all could cause contact angle and wettability changes.

Many physicochemical methods could improve the hydrophilicity but vary from different materials. Chemical methods include surface oxidation, grafting

modification, copolymerization, and surfactant modification. Physical methods include blending modification, high-energy radiation, and so on. For example, surface modification with collagen could be a feasible way to improve the hydrophilicity, and the incorporation of collagen on the polymer surface significantly enhanced the hydrophilicity and furtherly improved the attachment of fibroblasts [77]. Chemical modification to introduce diethylaminoethyl groups onto the polymer could also improve surface hydrophilicity and roughness, which subsequently enhanced the cell attachment and proliferation [78].

In summary, the methods for hydrophilicity improvement include two general ways: (1) surface roughness changes via physicochemical modification, which mainly changed the contact angle and wettability changes of the material surface, and (2) incorporation and coupling of hydrophilic components such as biological polymers and surfactants. The improvement of hydrophilicity will enhance cell/protein attachment, cell proliferation, and spreading on the scaffold surface, which promise better bone regeneration results.

1.4.4 Biodegradability

As we mentioned before, mechanical properties of scaffold play important roles for the structural support for bone formation. Although these polymers and inorganic components could optimize the mechanical structure for better bone regeneration results, the non-absorbable components such as metal or carbon could possibly cause cytotoxicity after long-term existence. Ideal scaffold should have proper biodegradability, and the absorption rate should be consistent with the bone formation rate [79]. After enough ECM are produced to provide structural support, the scaffold should be resorbed to prevent adverse effects.

After planted in vivo, the scaffold degradation suffered from biological degradation such as free radicals, enzymes, and cellular phagocytosis. Biodegradability materials which could be applied for bone regeneration include bioceramic, natural, and synthetic polymers. Synthetic polymers could also have good absorbability, but some degradation components have toxic and side effects. For example, the degradation products of PLGA are acidic components, which could increase tissue acidity and cause inflammatory responses. For bioceramics, they could be poor in toughness and flexibility, but the degradation products such as Ca^{2+} and PO_4^{3-} could deposit and promote bone formation [80, 81]. Natural polymers have excellent absorbability such as collagens, gelatins, and chitosan, but they usually have poor mechanical and processing performance.

Therefore, it's important to choose proper materials for scaffold design. Meanwhile, the degradation rate could be manipulated via changing the structures and composition of the polymers such as crystallinity and hydrophobicity. More importantly, the key point is to control the absorption rates and ensure that the scaffolds can withstand the appropriate external force before the new bone completely replaces the scaffold.

1.4.5 Biocompatibility

Biocompatibility is the most basic requirement for scaffold materials. It directly determined whether the nanomaterial could be applied to bone regeneration or not. It depends on the interaction between the materials and biological tissues, which includes two aspects: the host response and material reaction. For the host response, the most direct one is immunoreaction; the original components or subordinate degradation product may cause cytotoxicity or inflammatory reaction. Other negative responses for the host are mutagenicity and teratogenicity. For the material responses, the living system could have negative effects on the material including abnormal degeneration, corrosion, degradation, and absorption. The interactions between living cell and scaffolds is extremely complex. The biocompatibility reflected in the interaction between biological system and the materials, which could be affected by material components and their physicochemical properties. These factors will significantly affect cell adhesion, proliferation, spreading, biochemical activity and differentiation orientation, etc. The cell growth mode in turn directly affects the biocompatibility of the materials. Therefore, material modification and functionalization are the common ways to improve the biocompatibility of most materials.

1.5 Nanostructures and Scaffolds Applied to Bone Tissue Engineering

The design strategies of the scaffolds for bone tissue engineering should be biomimetic and simulate the biological environment of the biological bone matrix. As one crucial part of the strategy, optimal scaffold should be osteoconductive, osteogenic, and osteoinductive. Good scaffolds could incorporate and release growth factors to initiate and manipulate cellular activities and provide a suitable environment to stimulate bone repair and regeneration [82]. Since nanomaterials are applied to tissue engineering, nanoscale scaffolds significantly changed the tissue regeneration profile. With unique physicochemical properties, nano-sized materials have special biological properties to regulate cell responses like proliferation, migration, and differentiation. The strategy for bone tissue engineering scaffold design is to fabricate 3D structures with nanoscale and microscale effects, which is advantageous for cell attachment and differentiation. A variety of nanostructured scaffolds have been reported for bone regeneration. The major nanostructures are nanopattern [83], nanopores [84], nanospheres [85], nanofibers [86], nanotubes [87] and nanocomposites [88, 89]. Their fabrication, properties, interaction with stem cells, and osteogenic potentials are discussed in this part.

1.5.1 Nanopatterns

Nanopattern is one type of scaffold regulating cell responses via manipulating the surface nanotopography of the scaffold. The cellular behaviors on the scaffold could be different according to different surfaces [90]. Therefore, the architectural design and surface topography are very important for bone regeneration. Since researches have reported the guidance and regulation effect of surface morphology on the cell attachment, it's important to fully understand the nanopattern design.

Understanding how the nanotopography influence the cell attachment, morphology, and gene expressions is helpful to optimize the surface design of the nanopatterns. Tsimbouri et al. [91] investigated the role of the nanotopography in regulating the morphology and phenotype of MSC; the results demonstrated that the cell attachment, nucleus, and lamin morphology varied according to different nanotopographies. The interaction between stem cell and ECM could possibly directly or indirectly change the cell responses, which is called mechano-transduction. To furtherly understand the effect of mechano-transduction caused by surface topography, they used two nanotopographies, high intracellular tensions and osteogenic surface (near square 50, NSQ50) and self-renewal enhancing surface (square, SQ); the main differences between these two surfaces are the size of nanopits on the surface. The SQ nanotopography caused less phenotypical change, while NSQ50 nanotopography regulated osteogenic differentiation of MSCs.

In natural bone tissues, especially the bone during the healing process, nanodot-like topography with different intensity could be observed [92], which suggested that nanodot-like topography may be a feasible way for the scaffold to simulate the biological bone ECM. Kim et al. [93] fabricated nanopatterned substratum with different nanopillar intensity to design biomimetic bone tissue engineering scaffold. Among three different nanopillar pattern arrays (width to spacing ratio 1:1, 1:3, 1:5), nanopatterned substratum with 1:3 ratio showed the best bone mineralization results. The nanopillar pattern density also influences the attachment of osteoblast-like cells, which is a crucial step for bone regeneration on the scaffold. Besides, the results also demonstrated that attached cells spread more on the sparser nanopatterns. All these findings suggested that nanotopographical density could be regarded as a potential strategy for scaffold design. In conclusion, the nanopattern of the scaffold surface could regulate stem cell responses as a result of mechano-transduction. Cellular cytoskeleton contractility of the stem cell contributes to the mechanosensitivity of stem cell. Therefore, manipulating the nanopattern surface such as texture and nanopit intensity could be an efficient strategy for bone tissue engineering scaffold design.

1.5.2 Microspheres/Nanospheres

Nanospheres are characterized by their porous structures and controlled drug release. As we know, growth factors are crucial parts, and the sustained release of growth factors are encouraged for better bone formation outcomes. For conventional bulk scaffold, the initial burst releases of growth factors couldn't satisfy the requirements of long-duration release for bone formation. Therefore, nanospheres are expected to achieve controlled delivery of growth factor and extend their functional durations. Meanwhile, nanospheres have a large specific surface area, and cells could quickly attach and proliferate on the nanosphere surface in a short time.

The advantages of nanospheres in bone tissue engineering include sustained release for bioactive molecules, porosity optimization of bulk scaffolds, and injectable formulation scaffold design [85]. Nanosphere materials include polymer, ceramic, and composites. Polymeric microsphere/nanosphere is a common type of drug delivery systems since the 1970s. Natural polymeric nanospheres are favorable for bone regeneration because of natural biocompatibility and biodegradability. Common natural polymeric nanospheres include collagen, gelatin, chitosan, alginate, and so on. Natural polymer has cell recognition part, which is favorable for cell attachment. But mechanical strength might be a challenge for these natural polymeric nanospheres such as collagen. Synthetic polymeric nanospheres could provide proper mechanical properties such as PLA and PLGA. The biological activities of these synthetic materials could be well-controlled such as drug loading capacity and drug release kinetics. For example, Jeon et al. [94] fabricated heparin-incorporated PLGA nanospheres for fibroblast growth factor release profile investigation, the result demonstrated that fibroblast growth factor release from PLGA nanospheres remained for 3 weeks, and an initial burst release was observed. Although scaffold design could take advantage of the controlled growth factor release, most of these polymers have poor osteo-conductivity and osteo-inductivity. Inorganic microspheres/nanospheres could be alternative candidates for scaffolds with better mechanical properties such as CaP, bioglass, and other bioactive ceramics [95, 96]. However, the poor control of drug release restricted the practical application, and combination with other types of polymers will be favored for bone tissue engineering. For instance, Leeuwenburgh et al. [97] incorporated CaP nanocrystal with gelatin microspheres; these nanocomposites reduced the drug release rates and enhanced calcifying capacity, which combines the drug sustainability of gelatin and osteoinductive ability of bioactive CaP.

In summary, nanospheres have been extensively studied due to the potential drug delivery ability. They could be used as dispersed phase and building blocks. The most crucial roles for nanosphere in bone tissue engineering are vehicle for sustained drug release and enhancing the porosity of bulk scaffolds.

1.5.3 Nanotubes

Since firstly discovered by Japanese scientist Iijima in 1991 [98], this type of nanometer-sized hollow tube has been widely studied and applied to optoelectronic devices, nanosensors, nanocomposite materials, and biomedical area [99]. Since then, carbon nanotubes have always been a research hotspot because of their high stability and good mechanical and electrical properties. Besides, carbon nanotube material can increase the cellular adsorption rate and promote bone regeneration, so it has been extensively applied to bone tissue engineering [100]. Carbon nanotubes can be simply regarded as hollow tubes rolled up with graphite sheets, and they are separated into single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs). Generally, the diameters of SWNTs range from 0.5 nm to 10 nm, and the diameters of MWNTs range from 10 nm to 50 nm.

Carbon nanotubes are regarded as good scaffold material for the high strength, low density, and good biocompatibility. Unique tubular structure imparts carbon nanotubes special regulation effects on the cellular responses [101]. In 2006, Zanello et al. [102] studied the potential role of carbon nanotubes for scaffold materials, and this was the first time to prove that osteoblasts could grow and proliferate on carbon nanotubes. The cell morphology of osteoblasts was significantly changed, and obvious cell growth was observed. To figure out the potential mechanisms how carbon nanotubes influence the different cellular responses of the attached cells, Lin et al. [103] compared carbon nanotubes with GP; they reported that a large amount of protein adsorption on the surface of carbon nanotubes might be one of the mechanisms to promote the functional development of osteoblasts and predicted that carbon nanotubes are an osteoconductive material. The same evidence could be found in the study of Aoki et al. [104]: SaOS2 cells were cultured on the carbon nanotubes, and polycarbonate membranes (PC) coated carbon nanotubes and graphite. Carbon nanotubes showed better affinity for proteins and cells on the carbon nanotubes and showed better cell spreading, cell proliferation, and ALP activities. In all, high protein affinity of carbon nanotubes could be regarded as the reason of the enhanced cellular responses.

When we are deigning the materials for bone tissue scaffold, mechanical properties will be a very crucial factor. Carbon nanotubes have low specific gravity and high aspect ratio and can be repeatedly bent and twisted without damaging the structure. Therefore, carbon nanotubes are the best load-bearing reinforcing materials for the fabrication of composite materials with satisfying strength, light weight, and good performance. Compared with ceramic-based and metal-based materials, carbon nanotubes have a lower density, so it is easier to form high-strength, lightweight, and flexible scaffold materials. From the research of microtubule structure, it is found that the typical shape of single-walled carbon nanotubes is 0.5–1.5 nm in diameter and about 100–300 nm in length, which is very similar to natural bone, so it can mimic the collagen skeleton in geometric form that is beneficial to the deposition of inorganic substances such as calcium and phosphorus and then induces the nucleation and growth of hydroxyapatite.

Li et al. [105] investigated the osteoinductive effects of MWNTs on hMSCs; the adherence, proliferation, osteogenesis-related gene expression, and mineralization of hMSCs were significantly enhanced, and carbon nanotubes also enhanced the ectopic bone formation in vivo. But what are the different impacts on the cell behaviors between the single-walled nanotubes and multiwalled nanotubes? Hideki et al. [106] coated glass disks with SWNTs and MWNTs and treated MSCs with differently coated glasses. During the first 2 weeks, both SWNTs- and MWNTs-coated glasses promoted the early differentiation of MSCs to osteoblast. However, at the later stages of differentiation, higher osteocalcin expressions, mineralization, and calcium phosphate deposition were observed on SWNTs-coated glasses. Therefore, SWNTs might have better osteoinductive abilities than MWNTs in the late stage. The reasons for this difference might be the surface nanotopography and density of CNT; higher intensity promoted the osteogenic differentiation of MSCs. For the specially topological CNTs, the cell proliferation and osteogenic differentiation of MSCs could be enhanced. Specially patterned and aligned CNTs will enhance the expression of osteogenesis-related genes, which is a result of cytoskeletal tension in the aligned hMSCs [107]. Some other possible mechanism might be electrical stimulation from electrically conductive property CNT [108, 109].

Although CNTs exhibit potentially encouraging ability for osteogenesis, limitation also exists. Potential toxicity is one of the major nonnegligible problems for the application of CNTs in biomedical area [110]. After years of study, the cytotoxicity of CNTs is gradually discovered. The hydrophobicity, nonbiodegradability, and insolubility all contributed to the cytotoxicity of CNTs, which largely limited the biomedical application [111]. The existing chronic toxicity arise concerns for the long-term biocompatibility of CNTs after CNTs are applied to in vivo scaffolds. Evidence showed that CNTs might induce cellular DNA damages and apoptosis; the mutation frequency was twofold enhanced in comparison with the normal mutation frequency [112]. Some other cytotoxicity of CNTs include membrane damages, oxidative stress, and mitochondrial dysfunction [113]. After in vivo application, CNTs might cause organs damages such as oral, dermal, pulmonary, and systemic toxicities (immune responses) in a time- and dose-dependent manner [114–116]. Therefore, there is rare biomedical applications of pure CNTs due to their potential toxicities. Surface modification and functionalization were used to reduce the toxicity and increase the biocompatibility of CNTs [117]. Functionalization increased the solubility, hydrophilia, and solubility and subsequently changed their biological properties. The functionalization methods and components include surfactants, biomolecules, nucleic acids, and natural and synthetic polymers. Adsorption of serum proteins largely decreased the cytotoxicity of CNTs in comparison with pristine CNTs and change the cell interaction manner [118, 119]. Polyethylene glycol (PEG) was also used as surface modifications for many nanomaterials due to their excellent biocompatibility. Song et al. [120] studied the toxicity of PEG-coated CNTs on BMSCs, and the PEG imparts favorable biocompatibility to the CNTs. Natural polymers could also be used to functionalize CNTs. Sibel et al. [121] prepared nanotube-chitosan scaffolds, and the chitosan-MWCNT nanocomposites didn't cause significant cytotoxicity to the chondrocyte cells. In all, surface

modification of CNTs increased the dispersibility, biostability, and biocompatibility, which will improve the properties for wider biological applications.

1.5.4 Nanofibers

Like we mentioned before, the ideal scaffolds should be able to mimic the biological bone structures. As porous and hierarchical structures, nanofiber scaffold has been extensively studied for bone regeneration. The nanofiber scaffolds have similar morphological structures to the biological bone matrix and promote cell attachment and stem cell differentiation, which could be regarded as ideal scaffolds to provide structural supports. In terms of manufacturing techniques, nanofibers could be fabricated via several processes such as electrospinning, thermally induced phase separation (TIPS), self-assembling peptide nanofiber scaffold (SAPNS) [122], and bacterial cellulose (BC).

Electrospinning is a common technique for nanofiber scaffold fabrication. Polymer solutions are spun in the strong electric fields, the droplets at the needle will be transformed from spherical shapes to conical shapes and are continuously extended, finally forming fiber filaments. Under different conditions, manufactured polymer fibers could be different in diameters ranging from nanometers to microns. Due to the simple manufacturing equipment, low spinning cost, and abundant polymer sauces, electrospinning has become one of the main ways for effectively manufacture the nanofiber materials. A wide variety of nanofibers have been fabricated via electrospinning including organic, organic/inorganic composite, and inorganic nanofibers. Many factors could influence the spinning process including polymer property, shape of spinneret needle, needle-collector distance, and environmental parameters.

Materials used for electrospinning include natural materials (gelatin, hyaluronic acid, chitosan, collagen, etc.) and synthetic materials (polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL), etc.). The nanofibrous forms of these materials are polyporous with biomimetic structures. Over the past decades, the great potentials of electrospinning for bone tissue engineering have been demonstrated. For instance, Yoshimoto et al. [123] reported PCL nanofiber scaffold fabricated by electrostatic fiber spinning technique; PCL have good biodegradability, biocompatibility, and mechanical properties. Rat MSCs penetrated through the nanofibers, and much ECM was found after 1-week culture. Furthermore, the polymer fibers were covered by multiple layers of cells at 4 weeks, and mineralization and type I collagen could be found, which suggested great potentials of PCL nanofibers for bone regeneration. Other types of nanofiber scaffold also encourage the application of electrospinning nanofibers in bone tissue engineering [124].

Nanofibers fabricated with mono-component materials may not totally satisfy the requirement bone scaffold. Both natural materials and synthetic materials have some disadvantages. For example, natural materials might have insufficient mechanical strength, and synthetic polymer materials might lack bioactivity and

biocompatibility. Therefore, composite materials are more commonly used for electrospinning nanofiber design and fabrication. The combination of different types of materials could optimize physicochemical properties of nanofiber scaffolds. For instance, Yang et al. [125] incorporated chitosan into the PCL nanofibers; the chitosan-containing PCL nanofibers significantly enhanced the cell adhesion of MC 3T3-E1 cells. This kind of incorporation not only solved the insufficient mechanical properties of pure electrospun chitosan; it also changed the poor cell adhesion of pure PCL nanofibers. Linh et al. [126] fabricated polyvinyl alcohol/gelatin (PVA/GE) polymer composite nanofibers. PVA and GE are commonly used in biomedical area due to their biodegradable and biocompatible properties, but the PVA/GE scaffolds could be possibly dissolved in aqueous phases because of their hydrophilic and solubility. But after two components were cross-linked by methanol, the dissolution of the nanofibers in aqueous phases was significantly reduced. Meanwhile, the biological biocompatibility of the scaffold was promoted via GE incorporation.

Nanofiber scaffolds provided a good opportunity to optimize the scaffold design, but challenges still exist for clinical application of nanofiber scaffold. Further researches are required to manipulate the interactions between scaffolds and biological system, the pore size, mechanical properties, toxicity, etc. Furthermore, more researches and evidences are required to furtherly explore the clinical application.

1.5.5 Nanocomposites

Natural bone tissues themselves could be regarded as nanocomposite structures, which are consisted of inorganic HA and organic collagen fiber matrix ranging from nanoscale to microscale [127]. Single type of material couldn't totally simulate the biostructures and component of the biological bone. So nanocomposites could be regarded as potential candidates, which could mimic the bone matrix environment and biological properties [128]. As we discussed before, various types of materials have been proved to have osteoinductive properties, but polymeric composite materials are more extensively applied in bone tissue engineering because their physicochemical properties are more similar with the hierarchical and nanostructures of the natural bone [64].

Unlimited possibilities exist in the components for nanocomposite synthesis, but more common combination way for nanocomposites for bone regeneration is biocompatible polymer and bioactive inorganic nanomaterials [129, 130]. The polymeric polymers have many advantages such as good biocompatibility, easy modification, structural supporting, moldability, etc., which could play the role of organic collagen fiber matrix of natural bone. The inorganic bioactive materials could arise special bioactivity of the attached cells and optimize the biophysical and biochemical reactions, such as HA, tricalcium phosphate (TCP), calcium carbonate, and bioactive ceramic [128]. This kind of combination attracted much attention for biomimetic synthesis of bone-like nanocomposites, which combine the strength,

stiffness, and osteoconductive properties of inorganic components with the flexibilities, toughness, and biodegradability of organic phases [131]. Xin et al. [132] incorporated HA nanoparticles into the PMMA scaffolds to form HA/PMMA nanocomposites and found that the adherence and proliferation of osteoblasts are enhanced compared to single PMMA scaffolds. Similarly, Sharifi et al. [133] prepared nanocomposites composed of polyhexamethylene carbonate fumarate (PHMCF) and nano-sized HA; the addition of nano-sized HA improved the mechanical property of the nanocomposites and enhanced cell proliferation. There are many other studies that reported the HA-polymer nanocomposites, which changed the biological activities of the nanocomposites. Besides HA nanocomposites, other bioceramics such as TCP and calcium phosphates could also be incorporated in nanocomposites as bioactive components to optimize the mechanical properties [134, 135].

In all, nanocomposite scaffolds incorporate the advantages of different types of materials and are helpful to synthesize biomimetic scaffolds with structural and mechanical advantages similar with the real bone tissues. A wide range of combinations provide great opportunities to simulate the structure and morphology of native bones, but controllable bone regeneration and complex interactions between nanocomposites and bone tissue still require further studies.

1.6 Growth Factors and Molecular Pathways Involved in Osteogenic Differentiation and Bone Tissue Engineering

Over the past decades, it has been proved that nanomaterials could regulate cell response and facilitate cell migrations, proliferations, and differentiations. Besides stem cells and nanomaterial-based scaffolds, growth factors are also crucial in osteogenic differentiation induction of stem cells. As biological molecules, the growth factor usually has short half-life in living system and could be easily degraded. Meanwhile, the systematic application or sudden release of growth factors would cause side effects including edema, ectopic bone formations, delayed bone formations, or even carcinogenesis. Therefore, the scaffold achieves the sustained releases of growth factors and effective regulation of stem cells. The underlying molecular mechanism requires further exploration and understanding. The complete osteogenic differentiation includes the following process: bone progenitor cells differentiate into pre-osteoblasts and then form mature osteoblasts, and osteoblasts are mineralized in the extracellular matrix and become mature osteoblasts. Osteogenic differentiations of stem cells could be affected by physical, chemical, and biological factors and mediated by many regulatory factors and proteins. Therefore, research on relevant signaling pathways is essential for the development of bone regeneration scaffolds [27]. The participation of important signaling pathways in bone development has been confirmed by various studies. The role of some

important signaling pathways in osteogenic differentiation of stem cells and bone regeneration, such as the Wnt/β-catenin pathway, Notch signaling pathway, BMP/TGF-β pathway, and PI3K/Akt/mTOR pathway, which will be explained as follows.

1.6.1 Bone Morphogenetic Protein (BMP)

BMPs are the most widely used osteogenic growth factors, which could regulate stem cell proliferation and differentiation to osteoblast, thereby inducing new bone formation. Furthermore, BMP is also the only growth factor with ectopic osteogenesis ability. It is also the main factor that induces bone and cartilage formation and is expressed during body growth, endochondral ossification, and early repair of fractures and is also crucial in embryo growth and regeneration of the skeletal system. The two ways of bone formation, intra-membrane osteogenesis and endochondral osteogenesis, are directly induced by BMP. More than 40 subtypes of BMP have been identified and more commonly studied for bone regeneration which include BMP-2, BMP-4, BMP-6, BMP-7, BMP-9, and BMP-15. But the most studied is BMP-2, which has been approved by FDA for bone regeneration and has great potential in bone regeneration [136]. The regulation effects of BMP rely on two major signal pathways: Smad pathway and p38-MAPK pathway, which could induce osteogenic differentiation alone but also could collaborate with other growth factors to promote osteogenesis and bone formation. Take BMP-Smad signaling pathway, for example; endogenous or exogenous BMP signals bind with BMP receptor I and BMP-II on the cell membrane to induce phosphorylation of BMP-I and then interact with BMPs-specific Smad proteins. Phosphorylation of Smad proteins enter the nucleus and upregulate the expression of Runx2 and Osterix, which are two key factors regulating the osteogenesis process, thereby inducing bone formation [137].

BMP2 is currently the most studied and strongest osteogenic member of the BMP family. It's not only involved in osteogenesis but also in the key stages of embryo development and differentiation. It could also promote MSC to differentiate into osteoblast and has high osteogenic induction activity. BMP-2 also participate in bone healing process, Vivianne et al. [138] found that BMP-2 was mainly located in the periosteal layer and the endogenic expression of BMP-2 was essential for promoting fracture healing. For osteogenesis ability, the target cells of BMP-2 are undifferentiated mesenchymal cells and induce specific periosteum progenitor cells such as mesenchymal cells in muscles and around blood vessels, to irreversibly differentiate into cartilage and osteocytes. The application of BMP-2 in the therapy of bone fracture, trauma, and defects has achieved encouraging results in experimental research and clinical applications. The incorporation of BMP-2 into scaffolds promise good bone regeneration results. For example, Sun et al. [139] developed fibroin/nano-HA scaffold and conjugated BMP-2 into the scaffold through chemical combination; the controlled release of BMP-2 obviously improved the attachment

and osteogenic differentiations of BMSCs. Besides, it should be noticed that BMP-2 could also stimulate the proliferation of osteoclasts while promoting osteogenesis. In the later stage of bone healing, BMP-2 regulates osteoclast to directly or indirectly stimulate osteoclast differentiation and participate in the bone reconstruction [140, 141].

BMP-9 was firstly identified in the cDNA library of mouse liver [142]. It's involved in regulating cell proliferation, differentiation, and apoptosis, which cannot only regulate cell endothelial function and promote angiogenesis but also induce bone formation. BMP-9 is considered as one of the BMPs with powerful osteoinductive differentiation ability which is even better than BMP-2 [143–145]. It was also a major regulator of angiogenesis and chondrogenesis [146]. Since it has powerful osteogenic ability, BMP-9 could be used for bone regeneration. Zhang et al. [147] developed nano-HA-collagen-MWCNT composite scaffold carrying BMP-9 and found that BMP-9 scaffold could promote BMSCs to differentiate into osteoblast in vitro and induce more bone in vivo formation. Studies have revealed several regulatory pathways related to BMP-9 and osteoblast differentiation such as the classic WNT signaling pathways, Notch signaling pathways, mitogen-activated protein kinases (MAPKs) signaling pathways, the insulin growth factor 2 (IGF2)/PI3K/AKT signaling pathway, etc. For example, Cao et al. [50] suggested that Notch signal enhances early osteogenesis of MSCs induced by BMP-9 both in vitro and in vivo. The enhancement of Notch signaling pathway obviously enhanced the osteogenic differentiation induction ability of BMP-9 [148]. Tang et al. [149] investigated the roles of Wnt/β-catenin pathways in the BMP-9-mediated osteogenic differentiations of MSCs; they reported that Wnt3A and BMP-9 could significantly enhance the ALP activities in MSCs and they have synergistic effects on each other to regulate the osteogenic differentiations of MSCs. Downregulation of β-catenin expressions resulted in sharp decreases in osteocalcin expression stimulated by BMP-9. Li et al. [150] investigated the interaction between TGF-Smad and BMP-MAPK pathway; they found that BMP-9 induced osteogenic differentiations of MSC differentiation through the MAKP pathway and enhanced p38 and c-JNK. Besides these classical signaling pathways, other pathways also contribute to the osteogenic differentiations of MSCs regulated by BMP-9, such as insulin growth factor 2/PI3K/AKT signaling pathway and retinoid A (RAs) signaling pathways.

Other subtypes of BMP family such as BMP-4, BMP-6, and BMP-7 also participate in osteogenic differentiations of stem cells and bone formation. For example, study has demonstrated that if BMP-4 signaling was inhibited, obvious osteoporosis could occur, which suggested that BMP-4 signaling could be involved in regeneration and bone therapy [151]. The regulation effects of the BMP proteins incorporate with each other to synergistically promote the osteogenesis and bone formation.

1.6.2 Vascular Endothelial Growth Factor (VEGF)

VEGF is special in bone tissue engineering for their ability to induce neovascularization/angiogenesis. It is a type mitotic regulator of vascular endothelial cell, which participates in biological vascularization process, vascular permeability, and tissue inflammation. Besides angiogenesis regulation, it also participates in bone development, fracture repair, and promoting the proliferation and differentiation of bone-derived osteoblast [152]. There are two VEGF receptors Fltl and Flk in BMSCs; Fltl exists in the cytoplasm and nucleus, while Flkl is mainly found in the nucleus. After the Flkl or Fltl gene is deleted, the number of osteoblasts can be reduced, which indicated that both receptors are crucial for the differentiations of osteoblasts [153]. It could increase the osteogenic activity of osteoblasts and reduce osteoclast activity to promote bone formation and reconstruction. VEGF can directly regulate osteoblasts and increase the expression of osteoblasts ALP activity and promote their proliferation and differentiation and the formation of calcium nodules [154].

It was proved that exogenous VEGF can effectively promote the expression of early markers of osteoblasts [155]. After the BMSCs transfected with the VEGF gene, the levels of ALP, collagen I, and osteocalcin and the number of new blood vessels increased significantly [156]. It's proved that if the receptor of VEGF was blocked, the osteogenesis-related gene expressions and mineralization of MSCs would be reduced [157]. Generally, VEGF play important roles in bone regeneration at two aspects: (1) promote the angiogenesis and increase the microcirculation number to provide better blood supply for the bone tissue and (2) regulate bio-activities of BMSCs, osteoblast, and osteoclast to improve microstructures of new bone.

1.6.3 Basic Fibroblast Growth Factor (bFGF)

FGF is a group of homologous polypeptide family, and more than 20 subtypes have been discovered, which could be generally concluded into basic FGF and acid FGF, in which bFGF is more commonly studied. bFGF belongs to the heparin-binding growth factor family and could promote mitosis, cell growth, migrations, vascularization, wound healings, and tissue repairs. bFGF could promote the capillary to grow into bone grafts and accelerates the ossification of cartilage that requires blood supply, thereby increasing osteogenesis. Meanwhile, bFGF could promote the bone matrix synthesis of osteoblasts.

Zhang et al. [158] reported the acceleration of fracture healings by overexpression of bFGF; the acceleration effect was a result of the increase of VEGF expression and differentiation of MSCs to osteoblasts, which promoted angiogenesis and bone matrix production. Similarly, bFGF could also be used for tissue engineering scaffolds to achieve better bone regeneration results. Nakamura et al. [159] incorporated

bFGF into collagen scaffolds and applied the scaffold in the bone defect area; the controlled releases of bFGF significantly increased the bone volume and mineral content. However, the osteogenic effects of bFGF could act in time-dependent manners. Qian et al. [160] reported the time-dependent mechanism of bFGF on osteogenic differentiation of DPSCs; bFGF promoted osteogenic differentiation of DPSCs at the first week and inhibited osteogenesis in vitro and in vivo when it came to the second week. In recent years, the role of bFGF in osteogenesis and bone regeneration has attracted more and more attention and has broad prospects in the treatment of fractures and bone defects. But limitations also exist such as short half-life, which is the common limitation for most of the growth factors.

1.6.4 Insulin-like Growth Factor-1 (IGF-1)

IGF-1 is one type of growth factor rich in skeletal system and able to induce the osteogenic differentiation of MSCs [161]. It could also regulate bone growth through endocrine, paracrine, and autocrine including mediation of growth hormone and PTH-regulated skeletal activity. IGF-1 could regulate bone metabolism and stimulate osteoblasts to produce ECM proteins such as osteocalcin and collagen I, thereby promoting the bone matrix production and fracture healing. Under pathological conditions, MSCs expressing IGF-1 could promote the bone mineralization, thereby promoting fracture healing and improving the mechanical strength of fracture healing sites.

The loss of osteogenic potentials in the aging BMSCs was regarded as a critical issue for the bone deficit. Chen et al. [162] treated the aging BMSCs with high dose of IGF-1, and they found that the proliferation rates and osteogenic potentials of these aging cells were enhanced. The results suggested that IGF-1 could largely enhance osteogenic capability. Yuan et al. [163] investigated the gene expressions of MC3T3-E1 osteoblasts after the induction of IGF-1, the results of osteogenesis-related gene expressions (DMP1, PHEX, SOST, BMP2, RUNX2, OPN, and OCN) were obviously upregulated, and IGF-1 enhanced organic matrix production and bone mineralization. Several pathways are reported to participate in the IGF-1-induced osteogenesis such as ERK, JNK, and MAPK pathways [164].

IGF-1 could also enhance the osteogenesis via cooperation with other growth factors such as BMP. For example, Gustavo et al. [165] reported the synergistic effect of IGF-1 and BMP; they found that IGF-1 significantly enhances BMP-induced osteogenic differentiations of murine preosteoblasts and the ALP activity is higher than that of BMP-after combining with BMP-6. Bruno et al. [166] compared the osteoinductive potentials of IGF-1 and BMP-7 on MSCs; they found that BMSCs are more sensitive to the induction of IGF-1 and suggested the great potentials of IGF-1 to improve osteogenic differentiation of MSC.

1.6.5 Other Growth Factors Related to Bone Regeneration

There are some other growth factors which could possibly participate in the bone regeneration process such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF), and so on. TGF-β family is involved in regulating embryonic development, tissue regeneration, and immune system functions, which mainly consist of TGF-β1, TGF-β2, and TGF-β3. After binding with receptors, TGF-β could regulate cell growth, proliferation, differentiation, apoptosis, invasion, extracellular matrix synthesis, angiogenesis, and other biological responses. In terms of bone formation, TGF could accumulate MSCs to the bone resorption site and promote them to differentiate into mature osteoblasts via activating MAPK and Smad signals. For example, Yokota et al. [167] used TGF-β to induce MSCs and found that TGF-β could obviously enhance the expression of ALP in MSCs and induce osteogenic differentiations of MSCs in dosage-dependent manners. Manal et al. [168] studied the osteogenesis capacity of TGF-β1 with chitosan scaffolds, as the increase of ALP activities, mineralization, and osteogenesis gene expressions demonstrated that the combination of TGF-β1 and scaffold exhibits their potentials in bone tissue engineering.

PDGF could also contribute the bone formation and regeneration. It's a peptide found in platelets, which participate in neovascularization and stabilization. Currently, five subtypes have been found, among which PDGF-BB could enhance the proliferations and differentiations of osteoblasts and inhibit that of osteoclasts. The role of PDGF in osteogenic differentiation could be possibly controversial because it was reported that the inhibition of the PDGF receptors didn't significantly affect the osteogenic differentiation of hMSCs [169]. But many studies still suggested the positive effects of PDGF in the bone formation and regeneration. As an early inflammatory factor, the role of TNF-α in bone regeneration is enhancing proliferations, chemotactic migrations, and differentiations and influences bone formation [170]. As a type of co-growth factor, EGF could activate multiple downstream signaling pathways, which could regulate the biological activities of chondrocytes, osteoblasts, and osteoclasts [171].

The osteogenic differentiations of stem cell induced by various growth factors has been gradually clarified, but due to difference between artificial delivery and biological regulation in living system, more researches are needed to mimic the biological regulation effects of different growth factors, and much work are needed to achieve the precise control of these growth factors in bone tissue engineering such as time, concentration, the combination and ratio of different factors, and the order of priority of the growth factors.

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Chapter 2
The Application of Nanomaterial in Skeletal Muscle Regeneration

Yang Gao and Yunfeng Lin

Abstract In many clinical situations, muscle abnormalities in the human body, such as muscle weakness, trauma, myocardial infarction, and impaired striated muscle function, can lead to severe dysfunction. In recent years, cell therapy has aroused great interest in muscle tissue engineering therapy and direct myoblast injection and is considered as a very promising approach for skeletal muscle regeneration. Successful cell management is hampered. Multidisciplinary research work focuses on the in vitro culture and construction of muscle tissue, which depends on critical features such as cell orientation, multinucleated myotube formation, muscle fiber contractility, and density. Here we reviewed the research and application progress of nanomaterials in the treatment of skeletal muscle regeneration. We introduced different nanomaterials that have been widely concerned in recent years from the aspects of nanoscaffold materials and nanoparticles and systematically explained the design of nanomaterials from biological, chemical, physical, and mechanical aspects that affect the microenvironment of muscle regeneration.

Keywords Skeletal muscle regeneration · Nanoscaffold · Nanoparticle · Nanofibers

2.1 Introduction

Muscle tissue accounts for more than one-third of a person's body weight and has rightly attracted much attention from researchers. Although muscle tissue has a certain ability of regeneration, the treatment of volumetric muscle loss (VML) and severe myopathy still requires much manual intervention to promote the recovery of muscle structure and function. In many clinical situations, including muscular dystrophy, facial paralysis, traumatic injury, tumor resection, and so on, the impairment or loss of muscle function can cause severe physical discomfort. At present, the

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common treatments include autologous muscle transplantation and injection of cultured muscle cells in vitro. Although autologous healthy muscle tissue transplantation is a standard clinical procedure for the treatment of severe muscle injury, its shortcomings and limitations are also undeniable, such as the limited number and source of donor tissue, the need to open up a second surgical area, the function damage of donor area, and incomplete functional recovery. With the development of medical technology, the concept of minimally invasive treatment has been a beacon to guide the development of clinical technology in any treatment field, so is the development of muscle regeneration and tissue engineering. In recent years, cell therapy has aroused great interest in muscle tissue engineering therapy and direct myoblast injection and is considered as a very promising approach for skeletal muscle regeneration. Successful cell management is hampered. Multidisciplinary research work focuses on the in vitro culture and construction of muscle tissue, which depends on critical features such as cell orientation, multinucleated myotube formation, myofiber contractility, and muscle density [1]. Although the classical view of tissue engineering elements includes seed cells, growth factors, and scaffold materials, more and more studies have found the importance of the extracellular microenvironment to cell biological behaviors in recent years.

The tissue engineering is based on the patient's cells. With the help of guide by the scaffold material and stimulation by relevant growth factors, the cells cultured in vitro undergo proliferation and differentiation and would be finally transplanted back to the receiving area.

In cell-based skeletal muscle tissue engineering researches, commonly used cell types include satellite cells (SCs), myoblasts, and mesenchymal stem cells (MSC). C2C12 mouse skeletal muscle myoblasts are the most commonly used myoblast cell line [2]. Satellite cells are the most critical stem cells for skeletal muscle growth, maintenance, and regeneration, which were first suggested by Mauro 50 years ago [3]. In adult muscle, satellite cells remain inactive under normal physiological conditions that are sensitive to molecular triggers of movement, injury, or disease. Myoblasts formed by SC proliferation can differentiate and further fuse to form muscle fibers and repair damaged skeletal muscle tissue [4, 5]. SCs are less than 5% of the nucleus of muscle in healthy adult muscle tissue at rest, which limits the source of seed cells in muscle tissue and has also stimulated researchers to explore and promote SC muscle regeneration capabilities. MSCs can indirectly accelerate the activation of cells through paracrine signals, inhibit inflammatory responses, and thereby enhance the functional recovery of injured muscles [6, 7]. Compared to SCs, MSCs can be obtained from more extensive sources, including bone marrow tissue, cord blood, and adipose tissue [8, 9]. However, MSCs do not efficiently differentiate, and the ability to repair skeletal muscle is limited [10]. In muscle tissue engineering, first and foremost is sufficient cell expansion. However, in vitro expansion, cell stemness, and self-renewal ability are easily lost [11]. To overcome these challenges, researches in nanomaterials also tend to mimic the ECM microenvironment in skeletal muscle, including mechanical properties, biochemical cues, and electrical conductivity.

Researches on skeletal muscle regeneration have focused on scaffold materials and "growth factors." Especially in recent years, nanomaterials have been innovated continuously, and with their unique charm, they have promoted the application and

researches of nanoscaffold materials and nanoparticles in the field of muscle regeneration. Thanks to a similar size to biomolecules and systems, nanomaterials have been paid much attention to functional therapeutic or diagnostic material in recent years. A common feature of all nanomaterials is a great surface/volume ratio, so the advantage of using nanomaterials as carriers is that the material surfaces can be coated with lots of molecules. Widely studied nanomaterials in medical research include liposomes, dendrimers, gold nanoparticles, quantum dots, fullerenes, carbon nanotubes, nucleic acid nanomaterials, etc. Some of these nanomaterials have been approved for disease therapy of humans by the Food and Drug Administration. Nanomaterials can be used not only as a biological function but also as a carrier for transportation, targeting, imaging, and detection [12]. Stem cell research as a long-standing research field, not surprisingly, attracted a large number of researchers to explore the multidirectional application of nanomaterials in stem cells. The strong association has led to rapid advances in researches.

In this review, the application of nanomaterials in skeletal muscle regeneration in recent years was reviewed from the perspectives of nanoscaffolds and nanoparticles. The research and advancement of nanoscaffold materials mainly focused on the microenvironment around the seed cells, including biochemical clues, electrical conductivity, mechanical properties, and so on. The research of nanoparticles mainly focuses on the ability of nanoparticles to regulate the biological behavior of cells by entering cells and emphasizes the role of nanoparticles as a carrier in muscle regeneration. We hope to provide new perspectives for researchers in the area of nanomaterial and skeletal muscle tissue engineering.

2.2 The Research Progress of Nanoscaffold Materials in Skeletal Muscle Regeneration

The nanoscaffold materials for muscle tissue regeneration include two-dimensional and three-dimensional materials (Table 2.1). In addition to the good basic biocompatibility, nanomaterials should also mimic the microenvironment in vivo as much as possible and have the properties of promoting myoblast proliferation, migration, differentiation, and maturity. Compared with other tissues, skeletal muscle fibers also have electrical conduction and contractile-diastolic motor functions. Therefore, mechanical cues and electrical stimulation are also as important as biological and physical cues in the design of nanoscaffolds.

2.2.1 The Research Progress of Nanofiber Scaffold in Skeletal Muscle Regeneration

Nanofibers are one of the most commonly used cellular scaffolding materials in muscle tissue regeneration. They have attracted full attention because of the

Table 2.1 The nanoscaffold materials for muscle tissue regeneration

Number	Nanomaterials	Cell	Synthesis method	In vivo study	Research purpose	Reference
1	Nanoscaled fibrous PCL non-woven with oxygen functional hydrocarbon coating	C2C12 cells	Electrospinning and hydro-carbon plasma coating	No	The effects of the aligned topography and electrical cues of nanofibrous scaffolds on cell viability, cell number, myoblast orientation, and myotube differentiation	[1]
2	Graphene-PCL nanocomposite scaffolds	C2C12 cells	Electrospinning	No	The effect of graphene-PCL nanocomposite scaffolds on myoblast differentiation	[13]
3	Chitosan-PCL nanofibers	C2C12 cells	Electrospinning	No	The effect of chitosan-PCL physicochemical properties on the proliferation, adhesion, migration, and alignment quantification of C2C12 cells	[14]
4	Poly(ε-caprolactone) (PCL)/collagen-based nanofibers	Human skeletal muscle cells	Electrospinning	No	The effect of unidirectionally oriented nanofibers and randomly oriented nanofibers on cell adhesion, proliferation, and organization, including myotube orientation, diameter, and length of myogenic differentiation	[15]
5	Biodegradable block copolymer (DegraPol®)	C2C12 and L6 cells; primary human satellite cells	Electrospinning	No	Cell viability, adhesion, proliferation, and differentiation on coated and uncoated DegraPol® slides	[16]
6	PDA-modified nanofibrous scaffolds	C2C12 cells	Electrospinning	No	The combined effect of mussel-inspired polydopamine (PDA) surface functionalization and	[17]

					topographical cues on the behavior of skeletal myoblasts: myoblast adhesion, proliferation, and induced myotube formation	
7	PCL-PANi nanofibers	C2C12 cells	Electrospinning	No	The effect of nanofiber alignment, conductivity, and topographical cues on myoblast differentiation and myotube morphology formed on PANi/PCL nanofiber scaffolds	[18]
8	The electrospun PCL/PANi nanofibers	C2C12 cells	Electrospinning	No	The effects of the aligned topography and electrical cues of nanofibrous scaffolds on cell alignment, proliferation viability, myogenic differentiation	[19]
9	A 3D hierarchical structure consisting of the nanosized alginate fibers and the PCL strut	C2C12 cells	Electrospinning and 3D printing	No	The effect of the new hierarchical structure on mechanical stability, cellular ability, the alignment of myoblasts, and myotube formation	[20]
10	PCL nanofibers	Satellite cells	Electrospinning, plasma treatment and collagen cross-linking	No	The effect on cell proliferation and differentiation of satellite cells	[21]
11	PCL:D-ECM nanoscaffold	Satellite cells or muscle precursor cell	Electrospinning	No	The mechanical properties, bioactivity, and topographical cues to promote myogenic activity in primary satellite cells in vitro	[22]
12	Liquid crystalline scaffolds of peptide amphiphiles (PAs) nanofibers	C2C12-GFP myoblasts	Injectable gel	Yes	PA gel nanofiber alignment and stiffness direct the alignment, proliferation, and maturation of C2C12 myoblasts in vitro; degradation properties of the scaffolds in vivo; biomimetic scaffolds loaded with	[23]

(continued)

Table 2.1 (continued)

Number	Nanomaterials	Cell	Synthesis method	In vivo study	Research purpose	Reference
					growth factors enhance engraftment of transplanted MuSCs	
13	Peptide amphiphile nanofibers	Acute muscle injury in a rat model	Standard solid phase peptide synthesis chemistry	Yes	The bioactive properties of laminin-mimetic bioactive peptide (LM/E-PA) nanofiber on repairing acute muscle injury in a rat model	[24]
14	Nanofiber yarn/hydrogel core-shell scaffolds	C2C12 cells	Dry-wet electrospinning; photo-cross-linking	No	The effect on guiding myoblast alignment, elongation, and differentiation	[25]
15	Fe3O4/TiO2 hybrid nanofibers	Satellite cells	Sol-gel electrospinning	No	The effect on the cellular viability, adhesion, propagation, and spreading behavior of muscle cells	[26]
16	P3k/LAPONITE® nanocomposite gels	C2C12 cells	Injectable gel	Yes	The effect on adjusting extracellular microenvironment of myoblasts	[27]
17	Nanofibrous decellularized skeletal muscle extracellular matrix hydrogel	Primary GFP myoblasts	Injectable gel	Yes	Improving myoblast viability and maturation in vitro; improving cell survival and engraftment as a result of increased vascularization in vivo	[28]
18	GelMA-PdMGSMW hydrogels	C2C12 cells	GelMA hydrogel	No	The effect of GelMA-PdMGSMW hydrogels on adhesion, spreading, and differentiation of C2C12 myoblasts and contractility and metabolic activity of C2C12 myotubes	[29]
19	GelMA-CNT hydrogels	C2C12 cells	Dielectrophoresis	No	The effect on the viability, proliferation, differentiation, myotube formation of C2C12 cells	[30]

20	Zn/Al-LDH nanoparticle loaded with plasmid gene	C2C12 cells	Co-precipitation and ion exchange	No	High efficiency of sorting and release of drugs and gene in myoblasts	[31]
21	PEM films composed of HA and PLL	C2C12 cells	Microfluidics	No	The application of PEM films in revealing the optimal biochemical and mechanical cues necessary for myoblast biologic behaviors	[32]
22	Electroconductive nanopatterned substrates	C2C12 cells	Capillary force lithography; electron beam deposition	No	The effects of topography and electrical conductivity on cellular morphology and myogenic differentiation	[33]
23	Polymeric nanomembranes with CNT-Fn nanocomposite	C2C12 cells	Spin-coating and μCP techniques	No	The effect of polymeric nanomembranes with CNT-Fn nanocomposite on the cellular alignment, elongation, and differentiation	[34]
24	Fibronectin-gelatin nanofilms	C2C12 cells	Cell-accumulation technique	No	The effect of cell-accumulation technique in constructing skeletal muscle microtissues	[35]
25	PEG-CNT films	Human mesenchymal stem cells	The drop-drying method	No	The effect of PEG-CNT films on the skeletal myogenic differentiation of hMSCs in the absence of myogenic induction factors	[36]
26	PPy-PU nanocomposite	C2C12 cells	See the original text for details	No	The biocompatibility of PPy-PU nanocomposite on myoblasts	[37]
27	Poly(lactic-co-glycolic acid) nanoribbon sheets	C2C12 cells	Spin-coating and micropatterning techniques	No	The effect of PLGA nanoribbon sheets on cell orientation, proliferation, bilayer cell sheet formation, and myogenic differentiation	[38]

(continued)

Table 2.1 (continued)

Number	Nanomaterials	Cell	Synthesis method	In vivo study	Research purpose	Reference
28	Hierarchically aligned fibrous graphene-polysaccharide hydrogel films	C2C12 cells	Microfluidic self-assembly	No	The effect of nanocomposite fibrous hydrogel films on spreading, metabolic activity, and differentiation of C2C12 cells	[39]
29	BNNT functionalized myoblast/microfiber	C2C12 cells	See the original text for details	No	The effect of BNNT-functionalized 3D scaffold on myotube formation with the presence of BNNT	[40]
30	Mesoporous silica nanoparticle-based substrates with signaling modulators	C2C12 cells	See the original text for details	No	The efficiency for intracellular delivery of differentiation cues to promote myogenic differentiation	[41]
31	Cellulose nanowhiskers	C2C12 cells	Partial hydrolysis of cellulose; spin-coating	No	The effect on inducing contact guidance in skeletal muscle myoblasts and skeletal muscle myogenesis	[42]
32	Highly aligned 1D fullerene whisker (FW) scaffold	C2C12 cells	The liquid-liquid interfacial precipitation; the Langmuir-Blodgett approach	No	The effect on muscle fiber formation, cell adhesion, proliferation, and differentiation	[43]
33	Silk/melanin composite nanofibrous scaffolds	C2C12 cells	Electrospinning	No	The effect of silk/melanin composite nanofibrous scaffolds on myoblast proliferation, oxidative stress response, myogenic differentiation	[44]
34	PCE-graphene nanocomposites	C2C12 cells	One-step thermal polymerization	Yes	The effect of PCE-graphene nanocomposites on myoblast attachment, proliferation, myogenic differentiation, and promoting skeletal muscle regeneration	[45]

35	Nano-Au+HS complex	/	Electric nonexplosive method; conjugation via ultrasonic	Yes	The effect of the administration of nano-Au-HS on muscle development in vivo	[46]
36	Gold and gold-silver alloy nanoparticles	C2C12 cells	See the original text for details	Yes	The effect of AuNPs and Au-AgNPs on the proliferation, myogenic differentiation, and skeletal muscle tissue regeneration	[47]
37	PLL-coated BNNTs	C2C12 cells	A ball milling and annealing method	No	The interaction of PLL-coated BNNTs and myoblast uptake, cytocompatibility, and differentiation	[48]
38	MWCNTs	C2C12 cells	CVD method with catalyst (Ni)	Yes	The impact of MWCNTs with chemical surface functionalization on myoblast response	[49]
39	TiO_2 nanorods	C2C12 cells	One-step electrospinning technique	No	The effect of TiO2 nanorods on supporting myoblast adhesion, growth, and migration	[50]
40	Thermoresponsive nanofabricated substratum	C2C12 cells	Capillary force lithography	No	The fabrication of scaffold-free tissue-engineered construction with nanopatterned cell sheets	[51, 52]
41	Different carboxyfullerenes	C2C12 cells	See the original text for details	No	The cytoprotective activities of different carboxyfullerenes against oxidative-induced stress on postmitotic muscle cell	[53]
42	Silica nanoparticles	C2C12 cells	See the original text for details	No	The uptake and cellular trafficking of SiO2 nanoparticles during muscle regeneration	[54]
43	PLLA/ZnO nanocomposites	C2C12 cells	Solvent precipitation and compression molding	No	The impact of PLLA/ZnO nanocomposite dynamic surfaces on cell adhesion, morphology, proliferation, and differentiation	[55]

(continued)

Table 2.1 (continued)

Number	Nanomaterials	Cell	Synthesis method	In vivo study	Research purpose	Reference
44	Gold-KDEL peptide-siRNA nanoconstruct	C2C12 cells	See the original text for details	No	The delivery efficiency of siRNA in both undifferentiated myoblasts and differentiated myotubes	[56]
45	PEG-based protein nanocapsules	C2C12 cells, HeLa, HFF	See the original text for details	No	The application of PEG-based protein nanocapsules on the delivery and release of MyoD into myoblast cells	[57]

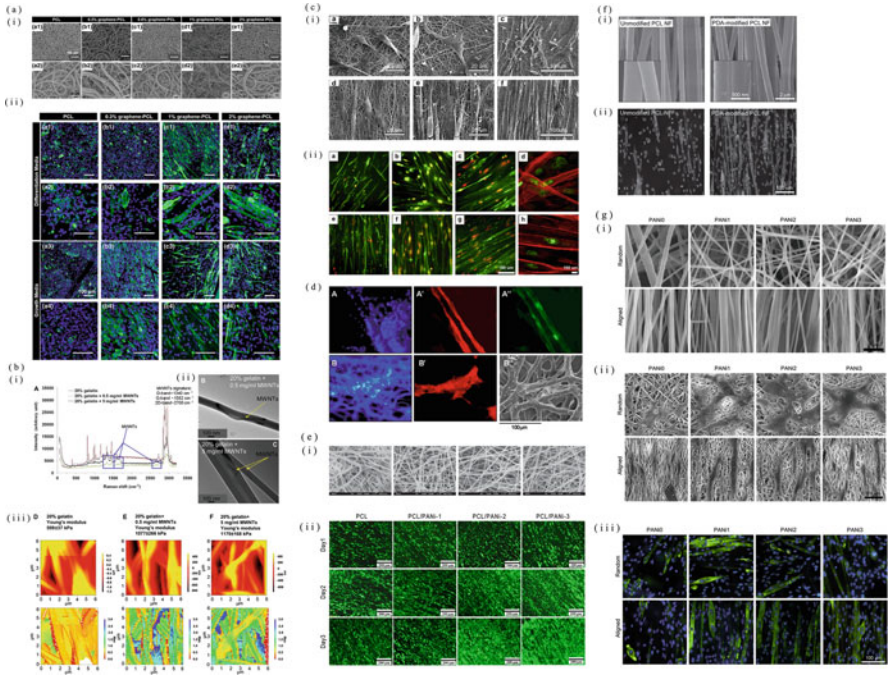

Fig. 2.1 Nanofiber scaffolds in skeletal muscle regeneration (a) (1) show SEM images of the fibrous PCL-graphene nanocomposite scaffolds prepared with different graphene concentration; scale bars of (a1–e1), 100 μm; scale bars of (a2–e2), 10 μm; (2) show the myoblast-instructive ability of nanocomposite scaffolds to induce multinucleated myotube formation even in normal growth conditions. All scale bars: 100 μm; adapted from reference [13]. (b) The characterization of 20% gelatin fibers encapsulated in different concentrations of MWNTs. (1) Raman spectra; (2) TEM images; (3) Young's modulus evaluation by AFM measurement; adapted from reference [58]. (c) (1) SEM images of hSkMCs on randomly oriented or aligned electrospun PCL/collagen nanofiber meshes. (2) Immunofluorescent staining images of hSkMCs on randomly oriented or aligned electrospun PCL/collagen nanofiber meshes; adapted from reference [15]. (d) Myoblast differentiation on electrospun degradable polyesterurethane membranes in differentiation medium (blue, nuclei; red, F-actin; green, MHC; gray, SEM micrograph). A represents C2C12 cells and B represents primary HSCs; adapted from reference [16]. (e) (1) SEM images of unmodified and PDA-modified PCL nanofibers (NFs); (2) myotube formation on unmodified and PDA-modified PCL NFs; adapted from reference [17]. (f) (1) SEM images of PCL/PANi nanofibers; (2) the morphology of C2C12 cell in growth media analyzed through SEM; (3) immunofluorescence staining for myosin heavy chain (MHC) after differentiation for 7 days; adapted from reference [18]. (g) (1) SEM micrographs after incubation in PBS for 2 weeks of pure PCL, PCL/PANi-1, PCL/PANi-2, and PCL/PANi-3 nanofibers; adapted from [19]

similarity to skeletal muscle ECM proteins (Fig. 2.1). Collagen is the main protein of ECM and exists in the form of nanometer size with a diameter of 260–410 nm [59]. Nanofibers can promote cell adhesion, proliferation, migration, and differentiation to obtain aligned cells. Nanofiber preparation techniques include the following three types: self-assembly method, phase separation, and electrospinning. Phase separation and self-assembly cannot produce oriented nanofibers, which

significantly limits their application in the regeneration of highly ordered and oriented tissues such as skeletal muscles. The most common technology for preparing nanofibers is electrospinning that has been studied and improved in associated researches in recent years.

2.2.1.1 Electrospinning Research Progress in Preparation of Nanofibers

Electrospinning is widely used in the production of nanofiber scaffold materials because of its simple preparation and the ability to prepare a variety of stable and personalized nanofibers [60, 61]. Degradable polymer materials pass through a needle with a high DC voltage source to form a charged solution jet. The polymer solution jet gradually stretches to form nanofibers during the solvent evaporation process and can form randomly oriented or oriented nanofibers. Oriented nanofiber materials are more satisfactory than randomly oriented materials for muscle regeneration [62, 63]. The degree and quality of fiber alignment and production rate vary by electrospinning systems.

Xu and coworkers have further refined the electrospinning methods to encapsulate cells in nanofiber hydrogels in a single step [64]. Compared with the traditional bulk hydrogels [65, 66], the new hydrogel nanofibers can retain bound water during electrospinning and prevent dehydration, which in turn maintains cell viability and stable proliferation. Neither the polymer precursor of electrospinning nor the electrospinning process affects cell viability. When the cells were electrospun with the scaffold material, the high hygroscopicity of the scaffold significantly prolongs the activity of the cells even in a dry macroenvironment. In order to study the actual viability of cells in electrospun scaffolds, the researchers prepared agarose gels via agarose gel electrophoresis and stained with living cells. After 3 days of culture, significantly more green living cells and fewer red dead cells in the refined electrospun hydrogel scaffolds were observed than the traditional bulk hydrogels of the same compositions. Seven days later, the differences between two scaffolds were even more pronounced. The underlying reason might be that the nanofibers can provide a larger internal surface, which in turn significantly enhances the interaction with the surrounding matrix and keeping cellular activity over a longer period of time. Further researches found that C2C12 cells could not only proliferate and spread in the electrospun nanofiber hydrogel in the 3D direction but could also maintain cell viability and proliferation capacity after passing the freeze-thaw cycle without additional antifreeze. This is beneficial for the use of electrospun living cells to produce spare tissue patches, which can be frozen and used when required by patients.

The chitosan (CS) membrane obtained by electrospinning combines the excellent properties of chitosan and nanofiber membrane and is an ideal substrate for soft tissue engineering, but the required acidic solvents significantly affect its biocompatibility [67]. The application of low acid content (0.5M) and disodium phosphate (DSP) as ionic cross-linking agents can improve the stability of nanofibers in water and neutralize the acidic pH of electrospun fibers after immersion [68], which overcome the limitation of biocompatibility. In addition, the improved process

avoids the impact of subsequent post-processing steps on the morphology of the nanofibers. C2C12 cells cultured on CS nanofibers indicated that the matrix arrangement could induce cellular orientation and promote skeletal muscle regeneration. What is more, the developed nanofibers also exhibit similar mechanical properties to the skin, nerves, and other biological soft tissues. However, since the electrospun membrane can only form small pores (1–3 μm) and lack high spatial connectivity between cells, the preparation method of nanofiber-based scaffold materials with larger pores still requires a further improved post-spinning process.

2.2.1.2 The Research Progress of Physical Cues of Nanofibers on Myogenesis

In recent years, there have been more and more studies using physical clues to obtain the corresponding stimulating effect on target cells, such as mechanical properties, surface topography, substrate hardness, and so on.

Some researchers have reported a non-biological magnetic hydrogel system that can simulate biological compression by providing external stimulation of magnetic and mechanical compression to promote the healing of damaged skeletal muscle tissue. Because of the ability to deliver electrical and mechanical stimulation, nanocomposites made of conductive nanomaterials are also used to enhance the regeneration of electrically excited tissues, such as graphene and carbon nanotubes [13, 69]. Graphene nanosheets with excellent mechanical, electrical, and thermal properties can provide conductivity for myoblast communication, nano-roughness for cellular attachment, and high specific surface area. However, at the same time, graphene nanosheets have slightly less flexibility and biocompatibility [70, 71]. Therefore, after combining with polycaprolactone (PCL), the graphene oxide-PCL nanocomposites have better biocompatibility and multinucleated myotube formation than graphene sheets alone. The researchers specifically investigated the effects of the physical characteristics of graphene-PCL nanocomposite scaffolds, including wetting, degradability, and mechanical properties on C2C12 myoblast differentiation into multinucleated myotubes when exposed to different concentrations of nanocomposite without any biochemical clues [13]. The results showed the physicochemical and mechanical properties of the nanocomposite scaffolds allow to be well controlled by adjusting the graphene concentration, and the graphene-PCL scaffolds of 1% and 2% graphene concentration showed more muscular myoblast-induced biological activity than other concentrations. The graphene-PCL composite scaffolds can significantly promote myoblast adhesion, migration, and proliferation and induce C2C12 myoblasts to form multinucleated myotubes. What is more, both composite scaffolds and degradation products own great biocompatibility. In general, graphene-PCL composite scaffolds have proven to be promising and potential skeletal muscle tissue regeneration cell-guided scaffolds.

To date, skeletal muscle constructed by tissue engineer has not been able to produce satisfactory high-density myotube arrangements [72, 73]. Skeletal muscle tissue engineer in vitro needs to reproduce nanoscale collagen fibrils and microscale

basement membranes of the natural extracellular matrix (ECM). Some researchers have constructed a nanosheet of chitosan-polycaprolactone nanofibers aligned with micron-scale chitosan ribbons to simulate the required muscle-forming environment [14]. Nanofibers and scaffold bands play a synergistic role in directing the orientation, interaction, and migration of C2C12 cell on this scaffold. The aligned nanofibers can mediate the cell arrangement, and the scaffold band can induce myotube cells to form tighter combinations. The expression levels of muscle-derived genes in the early and late stages were both higher than those in the control group. The above proves that nano- and microscale structural features can be designed to guide myogenic differentiation synergistically.

In muscle tissue engineering, myoblasts need to be arranged neatly on a scaffold to mimic muscle tissue more closely. ECM can provide mechanical support and regulate biochemical signals required for myoblast biological behaviors. One of the most important components of ECM is collagen fibers, which are often replaced by oriented nanofibers made by electrospinning in tissue engineering [74]. In addition, hydrogels are also commonly used for tissue regeneration, but the weak mechanical properties and poor electrical conductivity have limited their applications in regulating muscle regeneration. The combined application of various materials can concentrate on the advantages and improve mechanical, biological, and electrical properties [75]. For example, the mechanical properties and electrical conductivity of gel methacrylate gelatin (GelMA) hydrogels can be improved with the inclusion of carbon nanotubes (CNTs). Other researchers have also explored the role of multiwalled carbon nanotube-gelatin composite fibers in myotube formation [58]. After promoting the mechanical properties of fibers, the activation of genes related to mechanical transduction can be upregulated, which in turn upregulates the gene expression of FAK and myogenin and promotes myotube formation and maturation. Therefore, multiwalled carbon nanotubes can significantly enhance the formation of myotubes. The linear shape of the oriented gelatin fibers, the support of gelatin on cell viability, and the mechanical properties of multiwalled carbon nanotubes have jointly contributed to the orientation and differentiation of myoblasts and the formation of functional muscle fibers.

The bottom-up exploration of myoblast proliferation and arrangement, myotube formation, and morphogenesis of muscle tissue confirms the promising potential of nanofiber polymers in assembling cell and tissue structures from the nanoscale to the tissue level [76].

2.2.1.3 Biochemical Cues of Nanofibers on Myogenesis

In skeletal muscle regeneration, the differentiation of myoblasts into multinucleated myotubes is one of the vital assessment factors for the muscle induction potential of tissue engineering scaffolds. Myogenic differentiation is regulated by a range of biochemical factors such as growth factors, transcription factors, and myogenic regulatory factors [77–79]. This has inspired many skeletal muscle tissue engineering researches to provide biochemical clues to enhance myoblast differentiation. For

example, nanofibers can be functionalized by ECM proteins [15, 16]. The surface/volume ratio of polymer nanofibers is very high that surface modification can be carried out by adsorption or covalent bonding, especially surface adsorption [17, 80]. The adsorption of proteins on the surface of nanofibers is more than 16 times that of flat surfaces of the same area, which is more conducive to surface modification of nanofibers and regulation of myoblast behavior [81, 82].

Surface preparation of scaffold materials has become a key regulatory step in muscle tissue engineering nanoscaffold production because of its significant effects on cell behaviors. When designing biomimetic nanoscaffold materials of muscle microenvironment, surface chemistry and morphology are important factors in many parameters that need to be considered to affect cell-material interactions [83]. Noncovalent immobilization methods, including over-static, hydrophobic, and van der Waals interactions, are simple but highly dependent on the material chemical properties. The covalent binding methods require complicated chemical steps to achieve biological modification [84, 85]. Some small molecules with a catecholamine moiety can spontaneously form sticky polymers [86], such as dopamine and polydopamine (PDA). The investigation of the mixed effects of PDA-based functional modification and nanofiber alignment on skeletal myoblasts [17] demonstrated that surface modification with dopamine polymerization could contribute to myoblast proliferation and myotube fusion. Unmodified and PDA-modified glass substrates revealed similar cellular adhesion via further analyses of myogenic protein expression, myotube morphology, and fusion/maturity index but greater differentiation stimulation in myoblast cultured on PDA-modified substrates. What is more, on PDA-coated PCL nanofibers, myoblast fusion and MHC expression level both increased obviously, which suggests potential candidates of PDA-modified aligned nanofiber scaffolds for muscle tissue engineering.

2.2.1.4 The Research Progress of Electrical Conductivity of Nanofibers on Myogenesis

The upgrade of biological scaffolding materials is to design to improve the performance of various aspects of scaffolds. It is based on regulating the interaction between cells and the matrix of biological materials. Therefore, the scaffold material should be able to provide clues to mimic the natural microenvironment [87, 88]. Electrospinning nanofiber scaffolds have caused wide attention in tissue engineering-related researches in recent years, thanks to adjustable properties in geometric, mechanical, chemical, and electrical properties. Patel et al. have found that biologically active nanofiber scaffolds can enhance and guide extra neurite growth and skin cell migration [89, 90]. Composite scaffolds combining nanotopography and conductivity can help guide myoblasts to form muscle tissue structure and improve cellular maturation and function. One method of preparing conductive nanofibers is to integrate conductive nanoparticles into the polymer matrix, such as Au-NPs, carbon nanotubes, etc. [91]. The other is using carrier polymers and conductive polymers (CPs) to make composite polymerization, such

as polyaniline (PANI), polypyrrole (PPy), and polythiophene (PT). At room temperature, the emerald base (semi-oxidized form, PANI-EB) form is the most stable state of PANI and has the highest electrical conductivity. PANI-PCL composite nanofibers can be used to construct nanoscaffold to improve the differentiation of cultured myoblasts in muscle tissue engineering [18, 92]. Most researches on PANI-made biological scaffolds have concentrated on scaffold role modeling, material biocompatibility, cellular proliferation, and myogenic differentiation [93]. Therefore, some researchers have also explored the effects of gelatin-PANI composite nanofibers prepared by electrospinning on the maturation and function of muscle cells. Compared with pure gelatin nanofibers, composite gelatin-PANI nanofibers significantly promoted myotubes formation and accompanied by the improvement of myotube maturity [94]. In the colocalization of myotubes, intracellular tissues, dihydropyridine receptors, and ryanodine receptors, the expression level of proteins that are associated with excitatory contraction coupling devices, myotube contractility, and calcium transients was revealed to increase obviously. Such composite scaffolds, which combine topography and electrical conduction cues, can help guide the structure formation of skeletal muscle regeneration.

The nanoscale fiber morphology is considered to be one of the main structural features of ECM [95]. Electrospinning technology has become a practical approach to develop ECM-like fiber structure, because of lots of advantages in material production, including a large scale of nanofiber synthesis, easy control of diameter, and fiber orientation. Conductive polymers (such as PPy and PANI) can promote the proliferation and differentiation of skeletal muscle cells [92, 96], neurons [97], and cardiac myoblasts [98, 99]. Ku et al. prepared a polycaprolactone (PCL) nanofiber matrix containing conductive polymer polyaniline (PANI) to investigate the combined effects of electrical stimulation and topographical cues on cellular behaviors [18]. With the increase of polyaniline concentration, the conductivity of the blend nanofibers also increased. The researchers observed that C2C12 myoblasts seeded on PCL/PANI nanofiber scaffolds adhered to nanoscaffold and proliferated well, which indicated that the scaffolds own great biocompatibility. Myoblasts cultured on a randomly oriented nanofiber matrix were found a flat and multipolar morphology, but myoblasts cultured on uniformly oriented nanofibers adhered to a single nanofiber with a bipolar morphology. After further induced C2C12 cells to differentiate, the MHC positive area of myotube staining showed that the degree of myogenic differentiation depended on PANi concentration and nanofiber arrangement order. The electrical conductivity of the scaffold can coordinate with the oriented arrangement of nanofibers to promote myoblast differentiation, which emphasizes the important role of basal electrical activity and topographical cues in myoblast fusion and myotube maturation. The expression levels of myogenic genes, including myogenin, troponin, and MHC genes, also increased with the arrangement of nanofibers and the addition of PANi. Chen et al. applied PCL and PANi as blending solutions to impart biodegradability and conductivity, respectively, to nanofibers and prepared conductive nanofiber scaffolds with a highly oriented structure by electrostatic spinning [19]. When C2C12 myoblasts are cultured on ordered conductive nanofibers, the fiber arrangement as a topological clue leads to

directional cell morphology, and the electrical characteristics as electrical signals further stimulate the formation of multinucleated myotubes, thereby promoting myoblast differentiation. A fibrous scaffold with both guiding and conductive properties has a more satisfactory effect on stimulating myoblast differentiation than a scaffold with only one property. These researches suggest that PCL/PANi nanofibers have considerable application prospects in muscle tissue engineering scaffolds.

2.2.1.5 The Research Progress of PCL Scaffolds for Muscle Regeneration

Some researchers designed a new muscle tissue engineering matrix via applying plasma coating to modify the functions of electrospinning nanofibers and evaluated the influence of matrix properties on myoblast fate from several aspects of morphology, chemical surface composition, and mechanical properties [1]. The application of ultrathin oxygen functional hydrocarbon coating on electrospun polycaprolactone fibers with various diameters and orientations can enhance the stability of functional group formation and improve myoblast adhesion. The chemical characteristics and mechanical properties of matrices suitable for muscle regeneration in vitro can promote myotube maturation and allow cell contraction. Some researchers analyzed different C2C12 cells that are respectively grown on simple and functional substrates, including cellular viability, spatial orientation, cellular proliferation, myogenic differentiation, and myotube contractility. The results revealed that cell orientation was dependent on the basal structure and was most pronounced on parallel oriented nanofibers. Compared with pure PCL substrates, the changed surface characteristics, especially the carboxyl, carbonyl, and hydroxyl groups, are obviously beneficial for myotube differentiation. The results further demonstrate that the production of highly ordered contractile muscle tissues in vitro critically relies on the proper cell culture substrate.

Yeo et al. have used alginate nanofibers and microcapsules fibrillated with polycaprolactone (PCL) to obtain a hierarchical structure with a nanoscale morphology via combining the electrospinning technology and three-dimensional (3D) printing [20]. The improved process not only achieves enhanced mechanical stability but also can be used to induce myotube formation and promote the attachment and alignment of myoblasts. Cultured in a 3D hierarchical scaffold, the cellular orientation and myotube formation of C2C12 cells were obviously increased, which suggested the great potential of the 3D hierarchical scaffold as muscle tissue regeneration biomaterial.

Only when the mature myofibril is arranged in parallel to produce enough contractile force can the functional recovery of muscle obtained by skeletal muscle tissue engineering be satisfied [21]. Therefore, exploring suitable tissue engineering scaffolds to regulate the biological behavior of myoblasts has been the focus of research [100]. Nanotechnology promotes the creation of bionic nanopatterned scaffolds. Bionic nanopatterned scaffolds guide muscle regeneration and

reconstruction by simulating natural ECM. The nanoscale pattern of the nanoscaffolds can provide a broad surface for stem cell adhesion and functional differentiation. Compared with polystyrene, the differentiation and maturity of skeletal muscle satellite cells cultured on biodegradable polycaprolactone nanofibers are more satisfactory [21]. After enhancing collagen on nanofibers, the cell adhesion capacity of PCL nanoscaffolds increased obviously and is accompanied by a significant increase in satellite cell differentiation potential. After 2 weeks of culture on PCL nanofibers, the high levels of MyoD, MyH, CD34, and α-actin expression of myoblasts and low levels of M-cadherin and Pax7 expression are in accordance with the phase of myoblast activation and proliferation. These experimental results hint at the special role of nanofibers in inducing potential at the nanoscale, as well as the more applicable results of fixing collagen to nanofibers.

2.2.1.6 Other Nanofiber Scaffold Research Progress for Muscle Regeneration

Muscle loss of more than 10% of muscle mass is diagnosed as VML, which can lead to dysfunction. Regeneration scaffolds for VML are the focus of clinical treatment. However, because of the lack of suitable scaffolds to provide satisfactory biological and mechanical properties, there is no successful treatment method for VML. Although skeletal muscle has a remarkable regenerative capacity, VML injury is irreversible in human and animal models because it completely loses the essential regenerative elements, the substrate, and resident satellite cells [101]. VML injury showed obvious contractile tissue injury, persistent inflammation, extensive fibrosis, tissue structure changes, and dysfunction [101]. Previous studies have demonstrated that acellular extracellular matrix scaffolds (D-ECM) can be used to treat VML defects and promote muscle function recovery and blood supply after VML injury. However, the rapid absorption and limited support of DECM may cause excessive fiber deposition and even limit muscle regeneration [102–104]. By adding PCL, the mechanical properties and tensile mechanical properties of the D-ECM nanoscaffold can be enhanced. Moreover, in vitro experiments demonstrated that the PCL: D-ECM nanofiber scaffolds can jointly support satellite cell expansion, myofilament formation, and myogenic protein expression [22]. What's more, the myoblasts on aligned PCL: D-ECM scaffolds were revealed with higher cellular density and a more stretched and elongated morphology than non-aligned PCL: D-ECM scaffolds, suggesting a positive effect of aligned scaffolds on cell growth and survival. Aligned PCL: D-ECM nanofiber scaffolds can serve as a potential therapeutic method for VML.

Isolating enough satellite cells for muscle therapy remains a challenge. Therefore, to overcome the premature differentiation and the loss of the regenerative ability of stem cells caused by traditional culture conditions is also the focus of research on cell proliferation [11]. Based on supramolecular liquid crystals of peptide amphiphilic molecules (PAs), some researchers have designed a new cell delivery method to encapsulate cells and growth factors together into a unidirectional and ordered

muscle-like nanofiber environment [23]. This hydrogel substrate, which mimics the stiffness of muscle tissue, can maintain MuSC function in vitro and improve MuSC proliferation together with small molecular inhibitors of P38 mitogen-activated protein kinase [105, 106]. The stiffness of nanofiber templated PA scaffolds is related to the amino acid sequence, which affects the arrangement of cells in the macroscopic view. Because of the support of PA scaffolds on myoblast survival and proliferation and induction effect on myogenic differentiation and maturation, nanofibers can be aligned with endogenous muscle fibers in PA solution and assembled into scaffolds to form an in vivo delivery system. This unique liquid crystal has a number of advantages, such as muscle-matching rigidity, strong ability to retain growth factors, and biodegradation rates adapted to the timescale of muscle regeneration. Most importantly, the scaffold can improve the efficiency of cell implantation in damaged muscles without causing cellular damage when combined with growth factors.

Skeletal muscle laminin is the main ECM part of skeletal muscle tissue. It not only protects muscle fibers from external damage but also triggers satellite cell fusion to promote healing after injury [107, 108]. Therefore, by simulating the function and structure of laminin in skeletal muscle, a designed bioactive peptide (LM/E-PA) system can accelerate the activation of satellite cells [24]. LM/E-PA nanofibers can promote the differentiation of myoblasts in cell experiments. Injection of the bioactive nanosystem into the anterior tibialis muscle of rats with acute muscle injury can significantly promote activating satellite cells and enhance myofibril regeneration after skeletal muscle injury and shorten the time needed for functional and structural reconstruction. From the behavioral, physiological, histological, and molecular biological detections, all results supported the significant effect of injection of laminin-like self-assembling peptide nanofiber network without additional treatment on promoting myogenic differentiation and muscle regeneration. Except for muscle repair, the peptide material of LM/E-PA system also has clinical significance in the healing of injured skeletal muscle.

Some researchers have designed a core-shell composite scaffold consisting of a core of electrospun nanofiber yarns that mimics the arrangement of muscle fibers, and a photo-cross-linked hydrogel shell simulating connective tissue around myofibers [25]. First, aligned nanofibers are prepared with PCL, PANI, and silk fibroin (SF) mixture by dry-wet electrospinning. The nanofiber core is then encapsulated in the shell of photo-cross-linked hydrogel using a core-shell column and a sheet-like scaffold. Finally, the core-shell scaffolds were seeded with C2C12 myoblasts, and the changes in cell arrangement, migration, and differentiation were detected. The researchers found that the core-shell scaffold could promote the arrangement and differentiation of myoblast and create a 3D environment to provide mechanical protection and nutrient exchange in a large number of practical applications for skeletal muscle regeneration.

Good biocompatibility is the basis for research into the use of nanomaterials in tissue regeneration [109]. Titanium and its alloys are also widely used in dental implants and plastic surgery because of excellent biocompatibility, great chemical stability, and high mechanical strength. Some researchers have investigated the

interaction of nanostructured iron oxide and cells. For example, Fe_2O_4/TiO_2 nanofibers show good biocompatibility in muscle satellite cells, which can guide muscle satellite cells to adhere and proliferate, suggesting its effect on promoting muscle regeneration [26]. In particular, the small diameter (about 200nm) Fe3O4/TiO2 composite scaffolds can simulate the natural ECM well, providing potential in regenerative medicine and tissue engineering.

2.2.2 *The Research Progress of Nanohydrogels in Skeletal Muscle Regeneration*

There is no doubt that suitable biomaterials are very important for the cellular treatment of muscle damage and should be able to unleash the full potential of growth factors and stem cells. Hydrogels are favored because they can encapsulate, support, and protect cells [110]. More importantly, hydrogels can form composite materials with other materials to achieve the purpose of improving mechanical properties and chemical cues in a targeted manner. Further functionalized synthetic hydrogels can be achieved by conferring materials with various bioactive molecules or other biohydrogels to contribute to forming and arranging multinucleated contractile myotubes in vitro [111].

As a type of biological material, the injectable hydrogel can exhibit sol-gel transition after injection. Because of effectively entraining living seed cells and functional molecules, the injectable gel precursor solution can form hydrogel of the carrier, cell scaffold, and anti-adhesion material after injected into the body [112–114]. Injectable properties also give the hydrogels the advantage of being minimally invasive. Today, many researchers have developed different types of injectable gels, including polyethylene glycol (PEG), gelatin, alginate, hyaluronic acid, and so on. Unlike conventional hydrogels, PLGA-PEG-PLGA/lapotine nanogels are unique in the ability to absorb and retain biologically active molecules for the cells in the scaffold. To assess the effect of the ability of nanocomposite gels on three-dimensional tissue formation, Nagahama and his coworkers transplanted green fluorescent protein myoblasts and injectable nanocomposite gels subcutaneously into nude mice [27]. After 28 days, the gel obtained from the mice showed mature morphology and structure of muscle, which demonstrated the advantages of the self-replenishing ability of the nanocomposite gel.

For a variety of skeletal muscle disorders, skeletal muscle progenitor cell injection therapy is minimally invasive, not limited by the low cellular survival rate, so the success rate is not satisfactory. The main reasons for the limited survival are from four aspects: cell death caused by acupuncture, immune cell reactions, the insufficient blood supply to the host tissue environment, and deficiency of biophysical support for the viability of exogenous cells. Therefore, there have been studies using nanofibers and decellularized skeletal muscle extracellular interstitial gels to build a muscle-specific microenvironment and improve the activity and maturity of

myoblasts in vitro [28]. Decellularized skeletal muscle ECM hydrogel is characterized by a proteome similar to that of healthy muscle extracellular matrix and a nanofiber structure similar to that of natural extracellular matrix [115]. Even in vivo, the more favorable microenvironment formed by this decellularized injectable hydrogel is also beneficial to improve the implantation of skeletal muscle cells and accelerate tissue vascularization. Experiments in vivo and in vitro more fully demonstrate the importance of the tissue microenvironment when cells are delivered to skeletal muscle.

The mechanical and electroconductive properties of hydrogels have important implications for their applications in muscle tissue engineering, biomechanics, and biosensing. Palladium-based metallic glass submicron wires (PdMGSMWs) help to obtain higher mechanical strength and better conductivity of gelatin methacrylate (GelMA) gels [29]. The mixed GelMA gel has a better upregulating effect on the adhesion and diffusion of C2C12 cells. With the stimulation of PdMGSMWs, the formation, metabolism, and contraction activities of myotubes also increase [116]. The expression of β1 integrin gene of C2C12 cells incubated in GelMA-PdMGSMW gel was 300 times higher than that in pure GelMA gel. The potential mechanism may be that the combination of the morphological and electrical properties of PdMGSMWs can increase the adhesion sites for cell elongation, because C2C12 myoblasts are sensitive to the electrical conductivity of scaffolds and matrix morphological cues, and will exhibit better adhesiveness and spreadability under suitable conditions. In addition, the biocompatibility of the hybrid gel is significantly better than the original gel. This new GelMA-PdMGSMW hydrogel may be used for the development of functional materials for electronic-biological interfaces, drug screening, and tissue construction.

High-water-content hydrogels attract considerable attention in biological scaffold materials due to great biocompatibility and biodegradability [117]. However, in order to obtain more satisfactory multiple performances in muscle regeneration, it is necessary to overcome critical defects including insufficient mechanical properties, no conductivity, and lack of anisotropy [118]. Nanomaterials with conductivity have been widely used to develop hydrogel performance. For example, the addition of gold nanoparticles can increase the conductivity of alginate hydrogels to obtain higher electrical stimulation on myoblasts [116]. There are also research teams that vertically arrange carbon nanotubes (CNTs) in methacrylate gelatin photopolymerizable hydrogel (GelMA) via dielectrophoresis (DEP) [30]. As a simple and fast method, DEP can form a nanofiber network structure, which has better mechanical properties and anisotropic conductivity than GelMA hydrogels with randomly distributed carbon nanotubes. Further researches found that myoblasts could differentiate into more functional muscle fibers on vertically aligned carbon nanotubes than that cultured on randomly and horizontally aligned carbon nanotubes. The expression of myogenic genes and proteins in myoblasts after electrical stimulation was more pronounced. This electrically adjustable GelMA-CNT hydrogel can be used for drug screening, the development of three-dimensional electronic tissue materials, and the study of biological actuators.

2.2.3 *The Research Progress of Nanofilm in Skeletal Muscle Regeneration*

Layered double hydroxides (LDHs) are a class of biocompatible inorganic layered nanomaterials. The special physical and chemical properties brought about by two-dimensional layered nanostructure are favorable for the controlled release of cargo drugs. LDHS can not only improve the drug loading density, physical and chemical stability, and penetration capacity but also protect the drug from environmental pollution and premature degradation. Yazdani et al. focused on new applications of LDH nanostructures as gene/drug carriers [31]. They evaluated the cellular uptake capacity of LDH plasmid/gene (pCEP4/Cdk9) as a viable option for RNA and DNA transmission in cells. Zn/Al-LDH is an economical and straightforward synthetic method and a reliable alternative to the traditional extension method. The results of the MTT assay confirmed the less adverse effect of Zn/Al-LDH nanoparticles of 200–300nm on C2C12 cells. In the experimental group, 94% of the C2C12 cells were healthy with no statistical difference from the control groups. Further testing of proteins and genes demonstrated that the drug sorting and release efficiency of Zn/Al-LDH nanoparticles in C2C12 myoblasts were high, which suggested Zn/Al-LDH nanoparticles a capable carrier for cellular uptake and delivery of a gene.

The microenvironment surrounding stem cells owns a critical regulatory effect on stem cell differentiation. In order to artificially simulate the microenvironment required for stem cells, co-culturing the stem cells with associated surrounding cell type is one of the effective methods. The co-culture membrane used is essential for inducing enough differentiation, which is required to allow effective biological interactions between cells, while preventing physical contact with co-cultured cell populations [119, 120]. To meet these challenges, nanofilms and highly porous (NTHP) membranes have been developed in recent years to be 20 times thinner and 25 times more lacunar than properties of traditional co-culture membrane [121]. By changing the conditions and parameters of the NTHP membrane manufacturing process, the membrane pore size can be finely adjusted at the nanometer scale. Owing to the capacity to enhance active contact between diffusing bioactive molecules and co-cultured cells via the custom-made membrane, the NTHP membrane system has a more substantial effect on promoting co-culture stem cell differentiation than conventional co-culture methods. Another property of the NTHP membrane is thermal responsiveness. NTHP membrane can generate transferable cell sheets in response to temperature changes, which is beneficial for harvesting differentiated cell sheets while avoiding damage caused by enzymatic hydrolysis. Moreover, NTHP membrane can form multilayer cell sheets for implantation to obtain better therapeutic effects than single-cell sheets [122].

Natural ECM is an essential microenvironment for cell proliferation, migration, and differentiation. It provides physical clues, biochemical clues, and high anisotropy and plays an important role in regulating cellular biological behaviors and development [123, 124]. Polyelectrolyte multilayers (PEM) have proven to be

promising candidate nanomaterials that mimic natural ECM biomaterials. Surface concentration gradients of four different materials adsorbed on the proton exchange membrane are composed of hyaluronic acid and polylysine, including a fluorescent phase-locked loop, fluorescent beads, cross-linkers, and polyelectrolytes grafted with cell adhesion peptides [32]. It was found that the proliferation capacity of myoblasts increased with the increase of the RGD peptide gradient, and myoblasts would adhere and migrate along the hardness gradient. This suggests that a gradient of biochemical and physical cues for stem cells own the potential to simply and efficiently screen the ideal mechanical and biochemical conditions needed for specific cells. In addition to stem cell applications, long-range surface gradients can also be used for substance-cell interactions research based on multiple advantages, such as a wide range of biochemical or physical cues, low requirement for material quantity, and large numbers of cells for lower error. The versatility of proton exchange membranes makes this gradient production technology applicable to various fields such as biosensors and drug screening.

The effect of substrate hardness on cells was initially studied using polyacrylamide gel, whose hardness can be adjusted by changing the amount of bisacrylamide cross-linking agent [125–127]. Although other synthetic polymers have been later developed, such as polydimethylsiloxane gel (PDMS) [128, 129], alginates, collagen, chitosan, and agarose, these gels are usually very thick. Whether the developed mechanical sensitivity is suitable for nanoscale films needs further investigation. Polyelectrolyte multilayer consisting of positively charged and negatively charged polyelectrolytes is a promising new method for the design of functional coatings, among which, the proton exchange membrane does not require a cell pre-coating protein to form a nonspecific interaction between the extracellular membrane and the foreign matter [130–133]. The surface properties of different types of cross-linked proton exchange membranes with different chemical properties and their effects on myoblast adhesion and proliferation were investigated. Myoblasts would spread more and proliferate faster, form a larger number and better tissue adhesion structure, and synthesize more actin fibers and protein plaques on the hard membrane. The trend has nothing to do with the chemical property of membranes. In order to study the mechanical induction of myoblasts, nanomaterials with adjustable chemical and mechanical properties need to be developed. The proton exchange membrane can be used in in vitro biophysical research and designed as a functional biomaterial surface coating.

In addition to the functionalities of the basal surface and hardness [134, 135], the biological scaffolds of muscular tissue engineering are also affected by the morphology of the stent surface [136, 137]. Studies have shown that the topographic map of ECM is a powerful clue to identify cell shape, orientation, arrangement, and functional regulation, emphasizing the importance of scaffold topography [138, 139]. Therefore, mimicking the nanotopography of skeletal muscle's natural ECM is an important reference consideration when designing skeletal muscle regeneration nanoscaffolds. Well-designed surface topography can promote cell adhesion, migration, and differentiation and is proven beneficial to tissue differentiation and regeneration. Besides the topographical condition, electrical stimulation

also plays a vital role in enhancing myoblast differentiation. Researchers have used capillary force lithography (CFL) to make nanopatterned polyurethane acrylate (PUA) substrates, then coated a metallic layer of titanium or gold via electron beam evaporator, and finally developed a conductive nanopatterned matrix for enhancing myogenic differentiation and maturity [33]. The deposited metal layer can not only maintain the topographical characteristics of the substrate but also impart conductive properties. The nanopattern of parallel grooves and ridges with a width of 800 nm and a height of 600 nm can simulate the ECM collagen fiber bundles and promote the maturation of primary myoblasts [140]. Interestingly, for C2C12 cells cultured on a conductive matrix, the electrical conductivity significantly affects the size of myotubes, cell fusion, and myogenic gene expression levels. Calcium is of vital importance during the excitation-contraction coupling in muscle cells [141, 142] and can enhance the differentiation of myoblasts by regulating the phosphorylation and activity of multiple transcription factors [143]. When the blocking of L-type calcium channel resulted in the decrease of calcium level in C2C12 myoblasts, the differentiation process is significantly inhibited [144]. When skeletal muscle myoblasts are cultured on a conductive substrate, increased intracellular calcium levels are also considered to be one of the mechanisms that further promote myogenic differentiation.

The bio-excited substrate formed via combining electron beam evaporation and CFL has advantages of high conductivity and high morphological fidelity, and this manufacturing process is a low-cost, scalable, and repeatable method. Increased expression of important myogenic genes is induced by the conductive matrix, which suggests that matrix conductivity may have important application potential in engineering functionality and bionic skeletal muscle tissue and can be used to therapeutic tissue construct and in vitro drug screening.

Biodegradable polymer ultrathin film (nanofilms) is a new class of quasi-two-dimensional polymer biomaterials studied in recent years. Nanofilms have independent structures with thicknesses of tens to hundreds of nanometers and can be applied to tissue engineering scaffolds, skincare, artificial joints, tissue defect repair surgery [145], surface coatings [111], drug delivery systems, and so on [146]. Some researchers have prepared polylactic acid nanofilms with magnetic nanoparticles (MNPs) as biological scaffolds to deliver myoblasts to skeletal muscle and evaluated the effects on cell adhesion and proliferation activity [147]. The surface roughness of MNPs-nanofilms can affect the morphology of the cells on the surface, and increase myotube area and fusion index during myoblast differentiation. Magnetic nanofilm is a type of unique tissue engineering scaffold material in the tissue regeneration study, which has the prospect of forming ultrathin and ultra-flexible scaffolds.

Adding nanomaterials to tissue engineering scaffolds can further enhance the electrical, chemical, mechanical, and biological properties [75]. Free-standing cell sheets produced via thermally responsive cell culture dishes can be stacked to produce a three-dimensional multilayer structure to mimic the ECM [122]. Ultrathin polymer nanofilms are typically tens of nanometers thick and have unique interfacial and mechanical properties. Embedding multiwalled carbon nanotubes with adherent micropatterns into the large surface area of functional nanofilms can be used as

nanoscale clues to control cell morphology and size [34]. The cell adhesion micropattern on the nanomembrane allows the arrangement of C2C12 myoblasts, and the embedded fibril CNTs enhance cellular elongation and differentiation to produce functional muscle fibers. This indicates that the surface of the nanomembrane is a useful tool for studying cell-substrate interactions and can be used to guide tissue engineering and design hierarchically assembled tissue structures to develop flexible biological devices and regenerative medicine applications.

The myogenesis process requires the proliferation, differentiation, and fusion of muscle precursor cells (myoblasts) to form differentiated myotubes. The three-dimensionally aligned muscle fibers in complex skeletal muscles are surrounded by ECM [148]. The most commonly used three-dimensional muscle tissue construction methods are biologically mixing polymer scaffolds with myoblasts [149–151] or using thermally responsive polymers to form scaffold-free 3D muscle tissue via multilayer cell sheet [152]. Some researchers have also developed a new "cell accumulation technology" that uses a fibronectin-gelatin (FN/G) film of approximately 10 nm to encapsulate cells like artificial ECM and then deposits on the substrate and spontaneously self-assembles to form tissue-like structures. Since previous studies have confirmed that this approach can be used to successfully construct 3D cultures of human skin fibroblasts and mesenchymal stem cells [153], it can also build a three-dimensional skeletal muscle tissue model [35]. By changing the number of myoblasts, the thickness of the structure can be easily customized and adjustable, and a three-dimensional multinucleated myotube structure with a thickness of about 76 μm at most can be established. This new method has significance in the study of the rapid formation of three-dimensional muscle tissue and has potential in constructing human skeletal muscle tissue model in vitro.

Human bone marrow mesenchymal stem cells (hMSCs) can be obtained from a wide range of sources and can maintain self-renewal ability and multidirectional differentiation ability under certain stimulation conditions. Although hMSCs can also induce muscle-derived differentiation, the expression level of muscle-related proteins does not show noticeable changes, which indicates that only the muscle-derived differentiation pathway of hMSCs is stimulated. Hence, the achievable application of hMSCs in skeletal muscle tissue engineering also requires a more efficient approach to guide hMSCs into more controllable and repeatable skeletal muscle differentiation. Carbon nanotubes (CNTs) are a kind of cylindrical tubular structure composed of graphene sheets and have been a hot spot in scaffold material researches in recent years [154, 155]. In methacrylic gelatin hydrogel, myoblasts cultured on vertically aligned carbon nanotubes could differentiate into more myotubes compared with myoblasts on horizontally or randomly aligned carbon nanotubes. This inspired Zhao et al. to design a type of polyethylene glycol-linked multiwalled carbon nanotube (PEG-CNT) film to enhance hMSCs' myogenic differentiation [36]. Nano-surface roughness, ordered arrangement, high mechanical strength, and hydrophilicity of PEG-CNT film can directly induce skeletal muscle mesenchymal stem cells into myogenic differentiation and fusion, even lacking myogenic inductive factors. Since the PEG-CNT membrane supports the specific

differentiation of hMSCs into skeletal muscle myoblast cell line, it can be used as a promising nanomaterial for skeletal muscle repair.

2.2.4 *The Research Progress of Nanocomposite Materials in Skeletal Muscle Regeneration*

It is difficult for a single scaffold material to own comprehensive capabilities in various aspects such as mechanical properties, biocompatibility, and biological effects. Therefore, in the design of nanoscaffolds for muscle tissue engineering, it is necessary to combine the advantages of different nanomaterials to obtain better muscle regeneration effects.

The response of myoblasts to electrical stimulation has also prompted the research of conductive polymers in the field of tissue engineering scaffolds, such as polypyrrole (PPy). PPy is often used in thermoelectric applications with other biological materials to improve nanomaterial performance, including electrical conductivity and mechanical integrity [156]. Thanks to great biocompatibility and highly durable biomedical use, polyurethanes (PUs) have a good reputation in various medical applications. The mechanical elasticity of PUs and the electrical property of PPy in composite biomaterials can be combined to achieve electrical regulation in a mechanically stressed environment [37]. This kind of hybrid material can promote the electrical and mechanical interaction of myoblasts in the newly formed tissue and enhance the assimilation of tissue engineering constructs and hosts. In addition, the effects of PPy and PUs composites on cell compatibility and myotube formation were analyzed, and the results confirmed the potential of PPy nanoparticles and PU composites as electromechanical couplers for myoblasts in tissue engineering.

Via spin coating and micropatterning techniques, Fujie et al. have developed unique structures of microfabricated poly(lactic-co-glycolic acid) (PLGA) nanoribbon sheets, which consist of central PLGA nanoribbons and four-sided strips [38]. The unique nanostructure can promote the arrangement of myoblasts into double-layer cell sheets and then obtain a layered and assembled cell structure. What is more, the expression level of myogenic genes in myoblasts cultured on bilayer cell sheets is significantly enhanced, suggesting that nanoribbon sheets have research potential during the differentiation and fusion of C2C12 cells. The cell bilayer or assembled multilayer is guided by nanoribbon sheets' own potential in facilitating tissue engineering for regenerative medicine and drug screening applications.

In the design of skeletal muscle regeneration guidance materials, various key biophysical characteristics need to be considered, such as multiscale hierarchies [157, 158], calibration cues for contact guidance [159, 160], good adhesion and wettability of cell-matrix interaction profile, mechanical properties [161], and electrical conductivity [162]. In recent years, carbon-based materials have been used to

mimic skeletal muscle ECM, such as various forms of carbon nanotubes and graphene [163]. Graphene has strong mechanical properties, complex structures that are easy to process, charge carrier mobility, and high electrical conductivity [164]. Although graphene-polycaprolactone (PCL) electrospun nanocomposites have the potential to guide the biological behavior of myoblasts, a multiscale hierarchical structure and an alignment structure for contact guidance are deficient. Hydrogels have been extensively applied to scaffold material in skeletal muscle tissue engineering due to the ability to accurately control physicochemical properties and the easy integration of nanomaterials [165]. Combining graphene with polysaccharides (chitosan and gellan gum) can form a conductive graphene-polysaccharide nanocomposite fiber hydrogel film with a hierarchical and aligned fiber structure [39]. The addition of graphene can improve the properties of nanofiber hydrogels, including electrical conductivity, tensile strength, wettability, and toughness, but not change the film elasticity. C2C12 cells seeded on composite nanofiber hydrogel membrane showed enhanced migration and differentiation to multinucleated myotubes. The oriented fibrous membrane structure and enhanced electrical conductivity can guide differentiated myoblasts to form multinucleated myotubes. In general, by improving the fiber arrangement, mechanical properties, wettability, and enhanced electrical conductivity of the scaffolds, differentiation along the fiber direction into multinucleated myotubes of myoblasts can be promoted from various aspects. Therefore, graphene-polysaccharide nanocomposite fiber hydrogel membrane is also a kind of promising biomaterial for skeletal muscle tissue engineering.

The electrical signal response is the basis for the normal functioning of many important tissues in the body, including muscles and nerves. A research group invented an innovative, minimally invasive method based on piezoelectric nanoparticles (such as boron nitride nanotubes (BNNTs)) to stimulate electrically sensitive cells indirectly [166, 167]. This method can give cells "electrical-like" stimulation but avoids the negative effects of direct contact with the electrical stimulation source. Pressure waves produced by ultrasonic waves are optimal activators because of external controllability and good safety. C2C12 cells cultured with polylysine (PLL)-coated BNNTs can effectively differentiate and fuse into myotubes on 2D culture plastics and hydrogels [48, 168]. In a further designed 3D polylactic acid (PLLA) scaffold with BNNT function, C2C12 myoblasts can differentiate into multinucleated myotubes and uptake BNNT under ultrasound irradiation [40]. Examination of intracellular connexin 43 (Cx43) and myosin revealed that myogenic gene expression of C2C12 myoblasts cultured on 3D scaffolds was considerably higher than myoblasts differentiated on 2D scaffolds. These results verify the importance of the platform scaffold dimensions and models in vitro.

Studies on the effect of electrospun single-walled or multiwalled carbon nanotubes and polyurethane conductive materials under electrical stimulation revealed that the formation of skeletal muscle tubes depended on the morphological cues of electrospun scaffold in the lack of electrical induction [154]. Multinucleated myotubes formed on electrospun polyurethane carbon nanotube scaffolds after electrostimulation were obviously more than that formed when no current passed,

and the effect of electrical stimulation on myoblast differentiation depends on the conductivity of the scaffold material.

2.2.5 *Other Nanomaterial Research Progress in Skeletal Muscle Regeneration*

Nanoyarn is also used in muscle tissue engineering. Compared with nanofiber scaffolds, nanoyarn has higher porosity, larger pore sizes, and a regular arrangement of fibers/yarns [169]. Nanoyarn scaffolds can promote cell proliferation, muscle tissue development, and ECM expression in in vitro studies. After 1 week of culture on nanoyarn scaffold, myoblasts can penetrate and proliferate deeply. In the horizontal direction, the actin filaments of myoblasts are arranged in a straight line, and the myoblasts on the nanofiber scaffold are arranged irregularly. This new type of electrospun nanoyarn scaffold may become a promising tissue engineering sling for clinical research, such as pressure urinary incontinence sling materials.

Targeting the inhibition of Notch signals is an interesting method to improve stem cell tissue differentiation and maturation [170, 171]. G-secretase inhibitor (GSI) is an effective Notch inhibitor, but its application is limited due to side effects. Mesoporous silica nanoparticles can work as a capable carrier of GSI to dynamically regulate the Notch signal of myoblasts and obtain better muscle differentiation [41]. During muscle regeneration, myoblasts would proliferate, migrate, then exit the cell cycle that is associated with decreased Notch signaling activity and finally fused with each other to form a multinucleated myotube. Solid scaffolds composed of mesoporous silica nanoparticles allow GSI diammonium phosphate to be delivered intracellularly in a controlled manner to promote differentiation without affecting myoblast cell proliferation. In addition, mesoporous silica composite nanoparticles support multiple application routes for oral, intravenous, and topical applications. A wealth of data supports the potential of nanoparticle-mediated Notch regulation in myoblast differentiation.

Cellulose is a linear homopolymer of β (1-4)-linked D-glucose, which can be biosynthesized into a fibrous structure with different crystallinity [172]. However, the crystalline regions of cellulose fibrils are scattered with amorphous regions, and the total crystallinity is only about 50–90%. However, in the acid hydrolysis of cellulose, the amorphous region is preferentially hydrolyzed than the crystalline region. The phenomenon of incomplete hydrolysis leads to the formation of stable rod-shaped nanoparticles with a diameter of 5–20 nm cellulose nanowhiskers (CNWs) [173]. Thanks to the special shape and nanometer size of CNWs, C2C12 cells will form a more oriented morphology in response to the CNW surface, and the more highly oriented the CNW surface, the greater the degree of myoblast fusion [42]. Only after 4 days of differentiation on the oriented CNW surface, highly oriented multinucleated myotubes were found. The cell orientation phenomenon results from the adjustment of the adhesion orientation on the surface of the CNW,

which may be the reason for the induction of contact orientation in C2C12 myoblasts. After 4 and 7 days of differentiation, the protein expression of MHC and the transcription factor myogenin could be observed under a confocal fluorescence microscope. Although multinucleated myotubes have formed in both groups, the 、 myotubes on the surface of CNW have higher MHC expression, and fused myotubes are more and longer. The average feature height of the CNW surface is only 5–6 nm, which is the smallest feature of inducing contact orientation for skeletal muscle myoblasts to date. This explores and highlights the potential of nanomaterials for engineering oriented tissues such as skeletal muscle.

Due to good bioavailability and biocompatibility, carbon clusters, including fullerenes, nanotubes, and graphene, have been used for drug/gene delivery [174–176], bioimaging [176], photodynamic therapy [177, 178], and biosensors in numerous researches. The electronic system of carbon clusters can facilitate the adsorption of extracellular interstitial proteins and promote cell adhesion and differentiation of carbon clusters [179–181]. Fullerene is a zero-dimensional spherical structure with a variety of self-assembled structures, such as fullerene whiskers (FWs). After C2C12 cells were seeded on an oriented FW substrate for 24 h incubation, cellular adhesion could be observed and analyzed by actin filament immunostaining [43]. The development of actin filaments and vinca protein activities was consistent with the margins of FWs. The myoblasts grown on random FW scaffolds have similar morphology to the myoblasts of the bare glass substrate. But myoblasts grown on oriented FW scaffolds are relatively slender, and the cellular growth direction is highly related to the FW arrangement direction. Therefore, oriented FW can promote the elongation of myoblasts and regulate the cell growth direction. Analysis of the rate of myoblast division revealed that after incubation on FW scaffolds for 24–48 h, the number of cell adhesion increased significantly, and the growth rate also increased significantly. After differentiated for 10 days, C2C12 cells immunofluorescence staining of the nucleus and myosin heavy chain (MHC) revealed that FWs could stimulate myoblast differentiation and fusion and the formation direction of myotubes was heavily consistent with the arrangement of the aligned fiber bundles. However, the myotubes differentiated on bare glass were randomly fused. Further analyses of the gene expression of MyoD and myogenin after 10 days of differentiation demonstrated that the expression of the two genes within the cells on the aligned FW was increased, which suggested that FWs not only accelerate myogenic differentiation but also control the direction of myotube fusion to form great orientation and arrangement. Therefore, oriented FW scaffold overcomes the structural limitations of other carbon cluster materials and is suitable as a scaffold for skeletal muscle tissue regeneration.

Myoblasts are electrically active and can regulate the differentiation process with each other through electrically active biomaterial scaffolds. Therefore, when designing new biomaterial nanoscaffolds for skeletal muscle tissue regeneration, mimicking the extracellular interstitial structure of skeletal muscle has always been a research focus. Related conductive polymers, including pol (3,4-ethylenedioxythiophene), polypyrrole, polyaniline, and multiwalled carbon nanotubes (MWNTs), have been combined with electrospun scaffold polymers

and conductive bioceramics to promote muscle differentiation [182–184]. There are also problems with biocompatibility, toxicity, stent form, and unmanufacturability. Silk fibroin is a protein extracted from silkworm cocoons. Because of excellent biocompatibility, biodegradability, and ease of processing, silk fibroin has received wide attention in various biomedical, bioelectronic researches and tissue engineering. Melanin is a heterocyclic compound [185] and a natural polymer pigment that can protect against sunlight and free radicals. Some researchers have combined the advantages of silk fibroin and melanin to develop antioxidant and electroactive silk/melanin composite (SM) in skeletal muscle regeneration to solve the problems caused by blending synthetic and conductive polymer limitations of the stent [44]. The results of C2C12 cells confirmed that the composite scaffold SM showed strong antioxidant properties, which could help reduce oxidative stress and reduce reactive oxygen species in myoblasts. In in vitro experiments, SM scaffolds promoted the proliferation of C2C12 cells and induced the cells to differentiate into well-arranged high aspect ratio myotubes, highlighting the significance of their antioxidant and electrical conductivity in muscle tissue engineering.

2.3 The Research Progress of Nanoparticles in Skeletal Muscle Regeneration

Functional nanomaterials including precious metals [186], oxides [187], carbon nanotubes [188], and graphene [189] have been increasingly used in various fields, including tissue engineering, photothermal agents, catalysts, mechanical strengthening, etc. [190, 191]. Numerous studies have verified that nanoparticles can promote myogenic differentiation and myotube formation, which own medical application significance and therapeutic potential in skeletal muscle regeneration [192, 193]. Graphene oxide and its conductive nanocomposites have the capacity to enhance myogenic differentiation and the potential in skeletal muscle repair in vivo [45, 179]. And the arrangement of gold nanorods can effectively upregulate the differentiation and orientation of myoblasts [194].

2.3.1 The Research Progress of Au Nanoparticles in Myogenesis

Due to easy synthesis, size design, good biocompatibility [195], and extensive upgradeability [196], gold nanoparticles (AuNPs) have had a wide range of applications in medical treatment, including regenerative medicine, drug delivery, bioimaging, and disease treatment [197–199]. AuNPs can promote myoblast differentiation via the Wnt/β-catenin signaling pathway and the p38 MAPK signaling pathway [200, 201].

In order to improve the viability and differentiation of myoblasts, some research groups designed electroactive biomaterials that could stimulate skeletal muscle repair and regeneration by simulating the mechanical and biological cues of natural ECM, such as carbon-based materials and conductive polymer materials [13, 18, 202]. The electrically activated surfaces of conductive nanomaterials can effectively increase intracellular calcium levels and further promote myoblast differentiation. Therefore, Au nanoparticles and carbon nanotubes (CNTs) both have been applied to produce conductive nanocomposites in skeletal muscle regeneration [33]. In addition, Du et al. have also designed a new highly elastic and scalable poly(citric acid-octanediol-polyethylene glycol)-graphene (PCEG) nanocomposite, which has adjustable proper biodegradability and electrical conductivity applied for myoblast differentiation, myotube maturity, and skeletal muscle regeneration [45]. What is more, PCEG nanocomposites can be prepared through low-temperature cross-linking and simple thermal polymerization technology. PCEG nanocomposites own great abilities for skeletal muscle tissue regeneration, including high elasticity, stretchability, conductivity, biodegradability, and biocompatibility. As a high elastomer matrix, 9PCE polymer can provide bionic elasticity, and reduced graphene oxide (RGO) can provide electrical conductivity and higher mechanical properties. After the PCEG nanocomposite was implanted in rats for 4 weeks, obvious highly biocompatible subcutaneous tissue could be observed. Compared with PCE and poly (D, L-lactide-co-glycolide), the multifunctional PCEG nanocomposite significantly enhanced myoblast adhesion, cellular proliferation, myogenic differentiation, and skeletal muscle regeneration in vivo, which provided a new strategy for the development of multifunctional elastic nanocomposites with high biocompatibility.

Through acting on transforming growth factor families and fibroblast growth factor-2 (FGF-2), proteoglycans can regulate the reactivity of myoblasts in muscle differentiation [203]. The biological interaction between myoblasts and FGF-2 is also affected by extracellular heparan sulfate (HS). HS mediates the action of many extracellular ligands and is involved in a variety of cellular behaviors [204]. Au nanoparticles (AuNPs) have a larger specific surface area and more reactive property because of a larger proportion of atoms uncovered on the particle surface. This also leads to a strong affinity of AuNPs for sulfhydryl groups, which contribute to selectively and accurately binding to target substances and entering the cells as a carrier for cargo molecules [205, 206]. During the self-assembly process, AuNPs can form complexes with organic compounds via noncovalent bonds, such as HS. Some studies have compared the effects of nanogold, HS, and nanogold-HS complexes on muscle development, especially the number of satellite cells and muscle tissue [46]. After the same period of skeletal muscle regeneration, the quantity of muscle cell nuclei of the AuNPs-HS complex group was the largest, and the muscle tissue was more round and more developed. The experiments demonstrate the positive effect of AuNPs and HS on muscle development, and the combination of AuNPs and HS can further enhance the beneficial effect.

Due to good biocompatibility and controllability [196], AuNPs have gained widespread attention in multiple medical research fields, including drug/gene delivery, regenerative medicine, cancer treatment, and bioimaging [197, 198]. In addition,

the size of AuNPs also has good personalization and customizability. Therefore, Au-derived nanoparticles are excellent and widely used models for exploring the interaction between cells and nanoparticles [179, 194, 207, 208]. MHC is highly expressed throughout the differentiation process of myogenic differentiation. MHC protein immunofluorescence staining confirmed that compared with the control group, the number of myotubes in the Au-AgNPs group and the AuNPs group increased significantly [47]. The myotube diameter, myotube length, fusion index, and maturity index in C2C12 cells also increased significantly upon exposure to AuNPs and Au-AgNPs. Further examinations of muscle-derived genes also showed that Au-AgNPs and AuNPs efficiently promoted the myoblast differentiation through increasing the expression levels of MyoG, Tnnt-1, and MyoD. Based on the biological effects of Au-AgNPs and AuNPs, preliminary explorations of the regulatory mechanism were conducted. p38a MAPK is one of the critical signal pathways involved in the signal conversion from mechanical stimulation to biochemical information. After entering myoblasts, AuNPs and Au-AgNPs also give mechanical stimulation to C2C12 cells and activate mechanical sensitivity pathway (p38α MAPK), which finally enhance myogenic differentiation. The detection results of proteins and genes related to the p38α MAPK signaling pathway consistently showed noticeable increase. After adding pathway inhibitors, the C2C12 cells interacted with AuNPs, and Au-AgNPs cannot achieve stronger differentiation effect than the control group. Therefore, the p38 MAPK signal pathway is of vital importance in myoblast differentiation enhanced by Au-AgNPs and AuNPs. What is more, Au-AgNPs have a more obvious ability to promote muscle-derived differentiation and skeletal muscle regeneration than AuNPs in an in vivo model of mouse tibialis anterior muscle injury. This further confirms the potential of monodisperse Au-based nanoparticles in the regulation of myotube formation and provides a novel approach to promote skeletal muscle tissue engineering.

2.3.2 The Research Progress of Nanotubes and Nanorods in Myogenesis

Carbon nanotubes can improve the tensile strength, shape recovery, compressive properties, thermal conductivity, and electrical conductivity of the material. The mechanical character of carbon nanotubes-hydrogels is dependent on the number of carbon nanotubes in the hydrogel system. The nanofiber network combined with electroconductive hydrogel owns the specific potential to improve myoblast adhesion. Even biodegradable scaffolds that are made with 89% carbon nanotubes as the main component and chitosan as the secondary component have great biocompatibility [209].

As one of the popular nanomaterials in the field of biomedicine [210], boron nitride nanotubes (BNNTs) not only have very high Young's modulus but also show better chemical and thermal stability than carbon nanotubes. The interaction between

polylysine-coated BNNTs and C2C12 myoblasts of in vitro studies confirmed good biocompatibility and significant regeneration promotion effects of BNNTs [48]. When reaching a polygalacturonyltransferase concentration of 10 g/mL and a culture time of up to 72 h, BNNTs have excellent proliferation and metabolic activity on C2C12 cells, while apoptosis, necrosis, and membrane permeation are completely absent. qPCR gene analysis and Western blot protein analysis showed that with the presence of BNNTs, myoblast protein synthesis, myotube formation, and expression of constitutive myoblast markers such as MyoD and Cx43 all increased.

Carbon nanotubes are a kind of allotropic form of carbon and are rolled up by a single-walled (SW) or multiwalled (MW) graphene sheet. Carbon nanotubes are widely used as drug carriers due to their easy modification capacity [211]. Because of high tensile strength, carbon nanotubes are also used for improving the mechanical properties of scaffold material. Another outstanding feature of carbon nanotubes is high stiffness and reversible foldability, including high tensile strength of 150 GPa and high stiffness values of 1 TPa. Because composed of only carbon, carbon nanotubes have excellent biocompatibility, low toxicity, and immunogenicity and become ideal candidates for biomedical applications [212, 213]. Various carbon nanotubes all have a good effect on cell growth and proliferation. Multiwalled carbon nanotubes can improve cellular fusion to form myotubes and myofibers, which leads to a rapid muscle regeneration process observed near the implant material. After a mixture of multiwalled carbon nanotubes and mouse myoblasts was implanted into the gluteal muscles of mice, muscle tissue replaced temporary granulation tissue during intense tissue regeneration [49].

The rapid development of nanotechnology has stimulated the synthesis and research of a series of one-dimensional structures, including nanowires, nanorods, nanoribbons, and nanotubes. Due to excellent optical and electrical properties, titanium dioxide has become a hot material in various fields in the past few decades and is widely used in photocatalysis [214], gas sensors [215], and nanomedicine. For example, TiO_2 shorter than 100 nm can induce apoptosis in lung fibroblast and breast epithelial cell lines under ultraviolet A irradiation. Even for different concentrations of TiO_2 nanorods, myoblasts could adhere to TiO_2 nanorods and proliferate and normally migrate without apparent cytotoxicity [50]. Synthesized TiO_2 nanorods are a type of prospective nanomaterial in a variety of medical applications.

2.3.3 The Research Progress of Other Nanoparticles in Myogenesis

Lots of studies have explored the effect of cell dynamics on the uptake and distribution of myoblasts during the incorporation of nanocarriers [216]. Under a confocal fluorescence microscope and a transmission electron microscope, cellular uptake and intracellular distribution of liposomes, mesoporous silica nanoparticles, polylactic acid-glycolic acid nanoparticles, and nanohydrogels in C2C12 cells are

similar. In addition to three-dimensional nanomaterial scaffolds for cell proliferation and differentiation, Penland and coworkers have also gained inspiration in constructing scaffold-free tissue engineering constructs in vitro and developed a thermally responsive nanofabrication matrix (TNFS) to realize scaffold-free 3D tissue engineering [51]. Magnetic nanoparticle-embedded cells can be cultured on TNFS, and nanotopographic cues can be used to create aligned cell monolayers that mimic the structure of the natural cellular environment [52]. After separated, the complete cell monolayer can be guided by a ring or disc magnet to promote cell sheet transfer and form 3D scaffold-free spherical tissue. The first basis is using temperature-sensitive poly(nisopryl acrylamide) to perform multiple functionalizations of nanofabricated substrates. For example, controlled nanoscale terrain, such as nanostripes and grooves, can guide and control the construction of cell monolayers. The formation of highly organized anisotropic cell monolayers can be observed when cells are cultured on an anisotropic nanopatterned matrix. However, cell action potential transmission and contractility are highly anisotropic, which indicates that anisotropic nanopatterned matrices provide a strong guiding role in regulating cellular arrangement and function in in vitro myoblast culture [217]. Different from the application of specific active molecules, the kind of tissue engineering platform is universal, which can be not only easily applied to the production of tissue engineering constructs containing complex physiological structures but also used to study the functional relationship of tissue structure, drug screening, and regenerative medicine. Everything has two sides, and the shortcomings of scaffold-free tissue engineering method are obvious. Although the cell surface monolayer can be spontaneously separated by the change of the surface wettability of the matrix, it is still difficult to control and manipulate the released cell sheet, because the thin cell monolayer will roll in and cause the loss of anisotropic morphology. This will affect subsequent manipulation of the cell monolayer and require further improvement in scaffold-free regeneration technology.

Muscle satellite cells are the most basic and essential element of skeletal muscle regeneration but easily damaged by oxidative stress. Fullerene and its derivatives have a unique cage-like structure and have been widely studied and confirmed antioxidants or free radical scavengers. Particularly, carboxyfullerenes have been famous for great water solubility, biocompatibility, simple preparation, and controllable structure. Therefore, Liu et al. have explored the protective structural effects of carboxyfullerenes of different sizes on C2C12 myoblasts [53]. Among the six kinds of carboxyfullerenes (TF60, TF70, DF60, DF70, QF60, and QF70), QF70 can best avoid oxidative stress damage to myoblasts and significantly improve C2C12 cells activity without affecting myogenic differentiation of myoblasts, which paves more theoretical foundation for the application of carboxyfullerenes in the field of nanomedicine and muscle tissue engineering.

Factors affecting the toxicological effects of nanoparticles on cell internalization include cell type and applied dose [218, 219]. The great ability to enter cells of NPs at biocompatible doses has been utilized for cell therapies [220], cellular track, and drug delivery [221–223], such as Duchenne muscular dystrophy. Fundamental research on the internalization mechanism of nanoparticles and their cell fate is of

great significance for understanding the further functional mechanisms. During cellular fusion, myoblasts will induce apoptosis phospholipid serine and receptor BAI1 to promote the fusion of normal myoblasts with multinuclear myotubes. Therefore, apoptotic cells and related receptors that recognize phosphatidylserine (PS) play an important role in myoblast fusion during muscle repair, regeneration, and development. C2C12 myoblasts can take up fluorescent silica NPs based on energy-dependent mechanisms, mainly through large-scale cytosolic and clathrin-mediated pathways [54]. After differentiation for 7 days, silica NPs of C2C12 cells were still present in the vesicles of fused myotubes. Low-dose silica NPs can increase myotube formation by promoting myoblast fusion. Apoptotic myoblasts can interact with healthy myoblasts through the BAI1 receptor. Then Bai1 promotes the fusion of healthy myoblasts via the ELMO/Dock180/Rac1 signal transduction pathway. Therefore, identifying PS on apoptotic myoblasts can improve the fusion of healthy myoblast. Apoptosis induced by silica NPs can work as biochemical clues in skeletal muscle regeneration.

2.3.4 The Research Progress of Composite Nanoparticles in Myogenesis

Polylactic acid (PLLA) is a biodegradable thermoplastic with good biocompatibility and nuclear plasticity, which is widely used in tissue engineering [224]. To meet the demand for different tissue engineering, PLLA needs to be modified to improve its performance as a scaffold material. In nanomedicine, NPs have attracted the attention of researchers because they can work on the scale of biomolecules and own special interactions with cells [205, 225]. NPs can be used as carriers to deliver different drugs and cytokines, such as ZnO NPs. Zinc can promote myoblast proliferation and differentiation by activating the Erk/Akt signaling cascade [226]. Trujillo et al. loaded ZnO NPs into the PLLA matrix and uniformly dispersed on the surface to form a degradable system, which enhanced myoblast differentiation [55]. However, due to the slow release of zinc, the effect of promoting differentiation is not surprising, and further upgrades are needed.

The foundations of AuNPs as a drug delivery platform include flexibility in synthesis, low cytotoxicity, and functionalization and enhancing cell uptake [227, 228]. When assisted by various charged groups such as amines [229], carboxyls [230], polyethylene glycol (PEG) [231], DNA [232], RNA [233], peptides [234], and antibody [235], the cellular uptaking of AuNPs can be further improved and controlled in different cells. Polypeptides can promote cell uptake of Au nanostructures through receptor-mediated pathways, such as polypeptides with a C-terminal KDEL (Lys-Asp-Glu-Leu) amino acid sequence, which is a highly conserved sequence for protein transport to the endoplasmic reticulum [236]. The KDEL sequence is considered to be a retention signal of soluble proteins and transmembrane proteins on the surface of vesicles mediated by a Coat-Protein I

(COPI). Interestingly, KDEL peptides have unique advantages in designing delivery vectors due to avoiding lysosomal degradation [228]. Therefore, some researchers have designed a combination of KDEL polypeptide sequence and siRNA against NADPH oxidase 4 (Nox4) on the basis of AuNP as a delivery platform and delivered to C2C12 cells and differentiated myotubes in the hope of improving muscle disorder treatment, such as muscle-related atrophy or cachexia [56]. Although higher concentrations of AuNPs are cytotoxic, AuNPs have been found to have good cell compatibility at concentrations below 20 nM [237]. Through transmission electron microscopy and laser confocal microscopy, the cellular uptake and efficient transfection of Au-KDEL nanostructures by myoblasts can be clearly observed [234]. AuNP-mediated colocalization of KDEL and siRNA indicates that AuNP nanocomposite structures own stable and effective siRNA transfection in C2C12 cells. After 24 h of transfection, about 90% overlap between siRNA and fluorescence of KDEL indicates the high stability of au-KDEL-siRNA nanostructures. In addition to myoblasts, it is interesting to note the effect of delivering siRNA into C2C12 myotubes. Approximately 68% were found to be delivered to C2C12 myotubes, obviously higher than 30% of liposome transfection. But the SiRNA detected in the myotube is possible from myoblasts that fused into the myotube during the differentiation and maturation. After 24 h of transfection, 80% of siRNAs is localized in the endoplasmic reticulum of C2C12 cells, suggesting that endoplasmic reticulum is the main site of AuNP-KDEL-mediated nanostructure transmission. Whether it is undifferentiated myoblasts or differentiated myotubes, AuNP-conjugated KDEL peptides can promote the intracellular delivery of SiRNA, thereby avoiding the cytotoxic effect of using cationic lipid drug carriers. More experiments may be needed in the future to elucidate the mechanism by which siRNA is released from nanostructured complexes and escapes from the endoplasmic reticulum to the cytoplasm.

Controlling the level of specific transcription factors within cells allows reprogramming of cellular function and differentiation to guide cellular fate [238, 239]. However, the therapeutic value of delivering recombinant transfer factors to target cells is limited by the structural fragility of the transfer factors and the inefficiency of membrane transduction. To overcome these challenges, lots of vectors have been designed with different functions to increase the efficiency of the application of transcription factors, such as PEG. Polyethylene glycol monomers and degradable cross-linking agents can be used to synthesize PEG-nanocapsules, and the physical properties and release kinetics of the nanocapsules are optimized by adjusting the ratio of them [57]. MyoD is a recombinant muscle-derived transcription factor. Under confocal microscopy, it can be observed that MyoD is transported into the nucleus by PEG-nanocapsules to induce myogenic differentiation of myoblasts. When the concentration of PEG nanocapsules is lower than 5 mM, it shows good biocompatibility in primary cells without cytotoxicity. The maintenance of the integrity and activity of transcription factors is the basis for improving the efficiency of intracellular delivery, and nanoencapsulated MyoD can overcome the longer protease challenge. Lots of advantages of polymer nanocapsules, including easy preparation, good biocompatibility, effective delivery effects, and customization

with physical properties, make it a useful tool for delivering various recombinant TFs for medical treatment. The protein can be encapsulated in PEG-nanocapsule without special modification, and the cross-linking agent is degraded, and the target protein is released only inside the cell.

2.4 Conclusion

In recent years, abundant progress based on nanomaterials has been made in skeletal muscle regeneration. In the design of biomaterials, it is no longer only inspired from the interaction between materials and cells to promote the regulation of the biological behaviors of materials such as adhesion, proliferation, migration, differentiation, and fusion of stem cells. The biological role of nanomaterials in building microenvironment around stem cells also needs to be considered. For nanoscaffolds in particular chemical property, mechanical properties, and electrical conductivity of material are important aspects that can be improved during material design and can be incorporated into composite design. The application of nanomaterials in the treatment and regeneration of skeletal muscle is not just the nature of materials themselves but also can be used as transport carrier, controlled release carrier, and "storage box." Skeletal muscle does not function completely independently, which needs to interact with the blood supply, nerves, skin, immunity, and joints. Therefore, looking ahead, complex muscle regeneration may need to work in concert with different tissue regeneration. So the selection and improvement of nanomaterials can also pay attention to its role in the vascularization, nerve healing, tissue healing, and other aspects.

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Chapter 3
Application of Nanomaterials in Neurodegenerative Diseases

Weitong Cui, Wei Fu, Tianxu Zhang, Ronghui Zhou, Tao Zhang, and Yunfeng Lin

Abstract The characteristics of neurodegenerative diseases (NDDs) are the loss of myelin sheath and neurons, which worsens and becomes dysfunctional over time. Alzheimer's disease, Parkinson's disease, and Huntington's disease are among the most harmful brain diseases. Effective treatments for NDDs are usually unavailable because of the difficulty in obtaining therapeutic drugs. What's more, the blood-brain barrier that selectively blocks the passage of substances is a dynamic interface between the brain tissue and blood. The presence of this barrier is effective in preventing some (mostly harmful) substances from entering the brain tissue from the blood, but it also causes the traditional drug transport systems to be unable to provide connectivity patterns and sufficient cellular structural repair, which is critical to functional recovery in brain diseases. Nanotechnology uses engineering equipment or materials to interact with biological systems, that is, to control and reduce side effects while inducing physiological responses through stimulation or interaction with targets, thereby completely changing the treatment NDDs. The nanomaterials have advantages in structure and performance and are designed as carriers to cross the blood-brain barrier to target location. Magnetic nanomaterials, as imaging agents or nanoprobes, have played an active role in the diagnosis of NDDs. The nanomaterials in clinical applications have not achieved the expected results, but it has made a breakthrough innovation, which points out the future development direction and lays a foundation for the application of nanotechnology in NDDs.

Keywords Nanomaterials · Blood-brain barrier · Neurodegenerative diseases

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ALVv	Anterolateral ventricular volume
BBB	Blood-brain barrier
CI	Cerebral infarction
CsA	Cyclosporin A
CSF	Cerebrospinal fluid
HD	Huntington's disease
Htt	Huntingtin
IPSCs	Human-induced pluripotent stem cells
LITA	Liposome nanoparticles
MCAO	Middle cerebral artery occlusion
MEA	Microelectrode array
MSC	Mesenchymal stem cells
NDD	Neurodegenerative diseases
NFTs	Neurofibrillary tangles
NanoCsA	Nanoparticle cyclosporin A
PD	Parkinson's disease
PEG-AU	Polyethylene glycol-AU
PEG-PLGA	Poly(lactic-co-glycolic acid)
PLGA	Poly-D,L-lactide-co-glycolide
rHDL	Reconstituted high-density lipoprotein
SLNs	Solid lipid nanoparticles
SPIONs	PEGylated superparamagnetic iron oxide nanoparticles
TBZ	Tetrabenazine
TDNs	Tetrahedral DNA nanostructures
VMAT-2	Vesicular monoamine transporter 2
TH	Tyrosine hydroxylase
TQ	Thymoquinone
ZnO-NF	ZnO nanoflowers
ZnO-NP	ZnO nanoparticles

3.1 Introduction

Neurodegenerative diseases (NDDs), including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), are caused by the accumulation of misfolded proteins, and they share some similarities in synaptic abnormalities and neuron loss [1, 2]. And NDDs can worsen over time, resulting in dysfunction. NDDs are caused by a variety of factors, including oxidative stress, mitochondrial dysfunction, and immune inflammation. Blocking one or two

pathways does not significantly reduce overall neuronal dysfunction and loss. With the deepening of the research on NDDs, the use of the advantages of multi-approach and multi-target treatment has a good effect on improving the symptoms and regulating brain function. On the other hand, the pathological changes associated with the onset of NDDs are irreversible. When patients have cognitive impairment, the course of the disease is often in the middle stages. At this time, treatment can only slow down the development of the disease and cannot fundamentally reverse the damage of the neural network. The cause of NDD is still unclear, and NDD cannot be cured, which seriously threatens human health and daily life and places a huge burden on families and society. Although many theoretically effective drugs have been developed, their effectiveness is greatly reduced for the existence of the BBB (a dynamic interface between blood and brain tissue that selectively blocks substances) [3]. When various solutes in the blood enter the brain tissue from the capillaries in the brain, they enter more or less quickly, and some cannot even get through. The BBB can make the brain tissue suffer little or no damage from harmful substances in the circulating blood, so as to maintain the basic stability of the environment in the brain tissue, which has important biological significance for maintaining the normal physiological state of the central nervous system (CNS). But at the same time, this selective permeability of the BBB is also a huge challenge for the treatment of NDDs [4]. After the advent of the scanning tunneling microscope, nanotechnology was born. Research on nanotechnology focuses on the characteristics and applications of nanomaterials less than 100 nm [5]. Nanomaterials have superior properties in terms of size, morphology, biology, chemistry, physics, and characteristics [6, 7]. The drug delivery system of nanomaterials can overcome the BBB. This advantage can better assist in the diagnosis of neurological diseases [8]. The next section introduces the characteristics of nanomaterials and the BBB, as well as the advantages and challenges of nanomaterials used in NDD. It focuses on the introduction of nanomaterials as an effective method for the diagnosis and treatment of nonspecific diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD).

3.2 BBB

Lewandowski proposed the concept of BBB in 1900. Initially, the blood-brain barrier was thought to be a barrier composed of brain capillary walls and glial cells, which can prevent substances in the blood (mainly harmful substances) from entering the CNS uncontrolled [3, 9–11]. The intact basement membrane, the glial membrane surrounded by astrocyte feet, pericytes, continuous capillary endothelium, and the tight junctions between the cells constitute the BBB [12–14]. Pericytes are located in the microvessels around the capillaries and plays a role in regulating BBB and supporting structures (Fig. 3.1a). At the same time, the tight junctions between endothelial cells form a network that limits proliferation. Chemical stability is maintained by the interaction of peripheral neurons with astrocytes. In addition, the formation of tight junctions is closely related to astrocyte foot processes. In

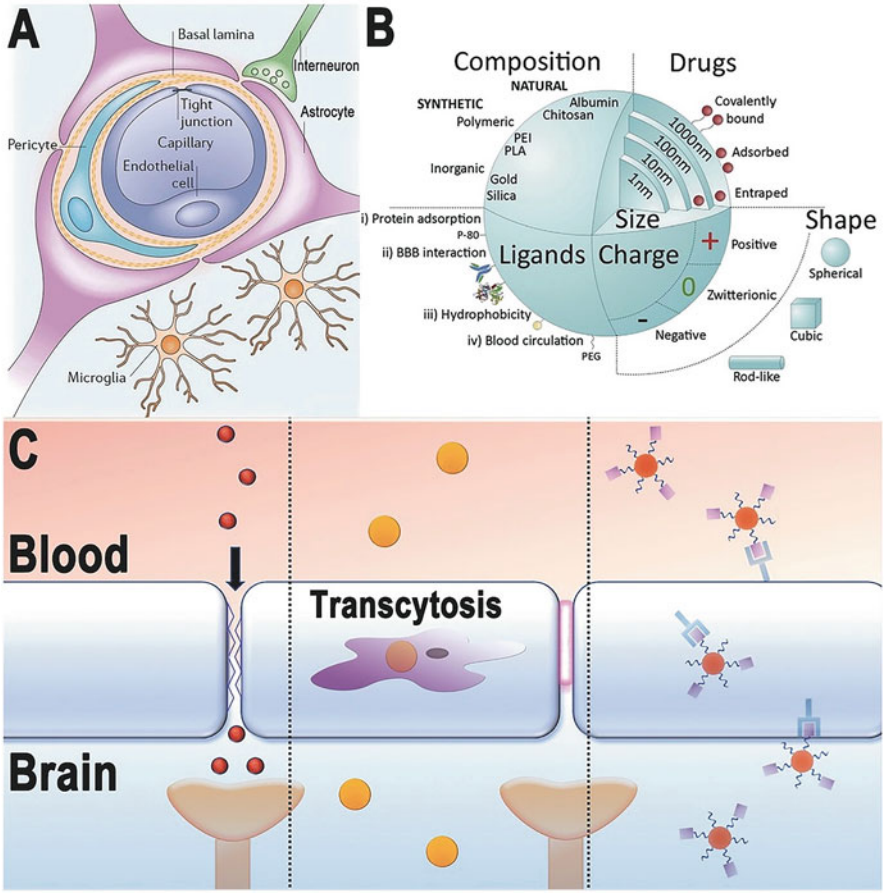

Fig. 3.1 (**a**) The schematic shows the structure of the BBB, which is formed by endothelial cells and surrounded by lamina and astrocytic perivascular endfeet. Pericytes and microglial cells are also presented. (**b**) The properties of nanocarriers such as type, charge, and shape among many others that affect the penetration and targeting of the BBB. (**c**) The various methods of transport of nanomaterials across the BBB for brain delivery. Copyright 2017, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

summary, the abovementioned cells interact with transport proteins and regulatory enzymes, which together constitute the densest barrier. As the maintainer and protector of CNS stability, the blood-brain barrier strictly separates the nervous system from the vascular system, allowing only certain small molecules to pass through. However, BBB also inhibits the entry of drugs through the same mechanism, hindering the effective treatment and diagnosis of neurological diseases [13, 15–17]. When degenerative diseases occur in the CNS, the presence of BBB blocks the entry of most theoretically effective drugs. This is due to acidity and alkalinity, fat solubility, and high molecular weight [15, 18–20]. Diseases of the

CNS can also affect the function and structure of the BBB, such as vascular cerebral hematoma. When vascular cerebral edema occurs, the endothelial cells of the cerebral capillaries are tightly connected and open, and the barrier permeability is significantly increased so that large molecules such as plasma albumin (molecular weight 69,000) can pass through the barrier. Severe brain injury leads to serious damage to the BBB, so serum proteins can also enter the brain through the barrier. As the damage is repaired, the influx of macromolecules into the brain stops first. The accelerated exchange of small molecules also disappeared after complete recovery, and the blood-brain barrier function was normal. Ionizing radiation, laser, and ultrasound can increase the permeability of the BBB.

The capillaries in the brain and their adjacent areas do have some distinct structural characteristics compared with that in other tissues and organs. Cerebral capillaries lack the pores common to capillaries, or they are few and small. Endothelial cells overlap with each other and are tightly connected, effectively preventing the passage of macromolecules from their junctions. Endothelial cells are also surrounded by a continuous basement membrane. Beyond the basilar membrane, the perivascular foot (end foot) of many astrocytes accounts for approximately 85% of the surface of the brain capillaries. This constitutes multilayer membrane structure, forming the protective barrier of brain tissue. In pathological conditions, such as vascular cerebral edema, the close adhesion between endothelial cells opens; as a result of the loss of endothelial cell swelling overlap, many macromolecular substances can be exudated with the plasma filtrate capillary, which will damage the stability of the environment in the brain tissue, causing serious consequences. A variety of solutes in the blood travel from the capillaries of the brain to brain tissue at varying speeds, and some cannot get through at all. This is determined by the degree of binding to plasma proteins, the lipid solubility, and hydrophilicity. The solute in the blood has to pass through the endothelial cells, which constitutes the cerebral capillaries, to reach the tissue of the brain. The endothelial cell membrane is bilayer structure with lipid as the base, which is lipophilic, and the fat-soluble substances are easy to pass through. Therefore, the lipid solubility of the solute in the blood determines the difficulty and speed of its passage through the barrier. The more fat-soluble the solute is, the faster it gets through the BBB and into the brain. Thus, some drugs of the CNS can be modified, according to this law, to make it easier to enter the brain to exert effect of drugs more quickly. For example, barbitone, a central anesthetic, with weak lipophilicity, is slow to enter the brain tissue. When transformed into phenobarbital and gaining lipophilicity, barbitone can enter the brain tissue through the BBB easily and soon play its hypnotic anesthetic effect. If morphine is transformed into diacetyl morphine, it is easier to pass through the lipophilic endothelial cell membrane, reach the brain tissue, and perform its analgesic effect faster. Carotenoids are fat-soluble pigments, but astaxanthin is the only member of the carotenoid family that can cross the BBB. Regardless of whether the solutes are positively or negatively charged,they form hydrogen bonds with the oxygen atoms of water molecules when they dissolve in water. The more charged the solute, the stronger its ability to form hydrogen bonds and the worse its ability to

pass through the blood-brain barrier. However, solutes such as water itself and glucose can enter the brain through the junction between endothelial cells and astrocytes due to their small molecular weight. Epinephrine and norepinephrine are difficult to get through the barrier because they are water-soluble and have many hydroxyl groups. Amino acids can cross the BBB, but amines have a harder time. Many compounds in plasma are bound to plasma proteins. Small molecules, such as hormones, cannot easily pass through the BBB when combined with plasma proteins and thus cannot exert their physiological effects. For example, nearly 99% of thyroxine binds to plasma protein and less than 1% is free. It has been proved that free thyroxine can readily enter the interstitial fluid of the brain. Thus, any drug which is able to prevent thyroxine from binding to the plasma protein can easily increase the amount of free thyroxine in the plasma, resulting in increased dose that passes the barrier.

In general, cell transport function is closely related to the lipophilicity and molecular weight of the transported substance to a large extent. Due to the effective efflux pump, although some drugs are lipoproteins, the drugs eventually return to the blood spontaneously [21, 22]. In addition, due to the tight connection between the cellular pathways and endothelial cells, it is difficult for large molecules to reach the brain to function. Therefore, a material that can overcome the selective permeability of the blood-brain barrier needs to be developed to successfully deliver the drug to the lesion [4, 23, 24].

3.3 Nanomaterials

As one of the emerging technologies with the greatest market application potential, the potential importance of nanotechnology is beyond doubt. The broad scope of nanotechnology includes nanomaterial technology, nanometer processing technology, nanometer measurement technology, and nanometer application technology. Nanomaterials have certain uniqueness. When the scale of nanomaterials is small to a certain extent, quantum mechanics must be used instead of traditional mechanics to describe their behavior. The reason why nanoparticles differ from large chunks is that their relatively large surface areas, known as ultra-microparticles, are covered with stepped structures that represent unstable atoms with high surface energy. Such atoms bond readily with foreign atoms and provide a large surface of reactive atoms due to particle size reduction. According to the content and characteristics of the research, the development history of nanoparticles can be divided into three stages after the advent of nanoparticle materials in the 1970s. It was divided into the first stage before 1990. In this stage, the research is focused on studying different methods of preparing nanoparticle powders in the laboratory, exploring the advantages of nanomaterials, and studying the evaluation methods. The research object is usually called nanocrystals or nanomaterials and is limited to single-phase materials. In the second stage, how to make full use of the physical and chemical properties of nanomaterials is the focus of research in this period. In the

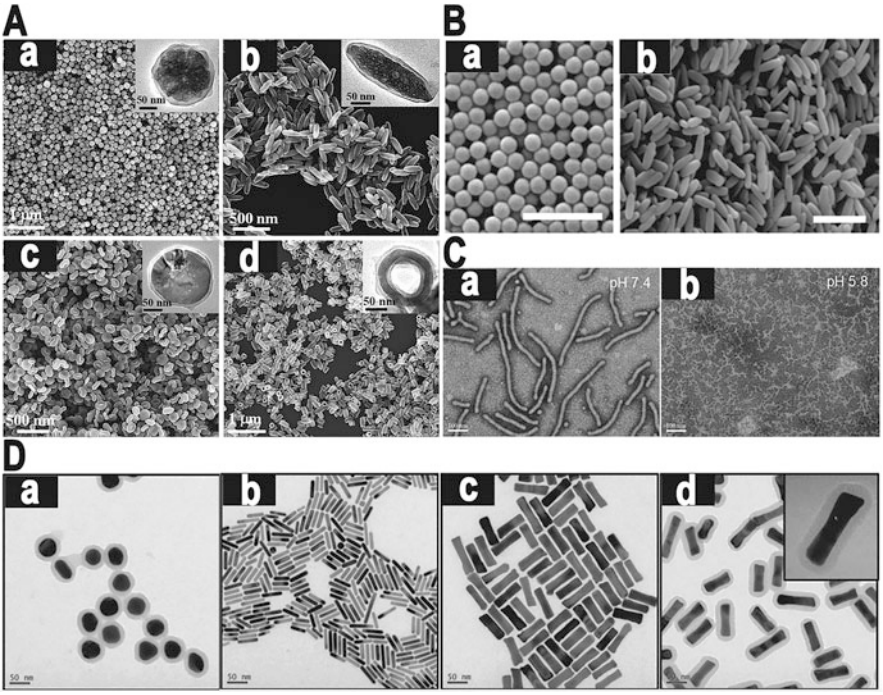

Fig. 3.2 Characterization of nanocarriers in several shapes. (**A**) FESEM images of different shapes of Fe3O4: (a) sphere, (b) spindle, (c) biconcave, (d) nanotube. (**B**) SEM images of polystyrene spheres (a) and elongated particles stretched from the 200 nm spheres (b). Scale bar, 1 μm. (**C**) TEM images of nanocarrier at pH 7.4 (a) and 5.8 (b), scale bar = 100 nm. (**D**) TEM images of morphologies in AuNRs (a) and AuNRs (b)–(d) at different steps. Copyright 2017, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

third stage (from 1994 to the present), the focus of nanomaterial research has shifted to the synthesis of nano-assembly systems, artificial assembly, and nano-structured material systems. This material is known internationally as a nanoscale mode material or a nano-assembly material system. Such systems are based on nanomaterial units, including nanoarray systems and mesoporous assembly systems. Specifically, nanoparticles, nanowires, and nanotubes are arranged in a multidimensional space to form a system. Fine-grained nanomaterials have many special properties such as macroscopic quantum tunneling effect, quantum size effect, dielectric confinement effect, surface effect, and volume effect compared with traditional solid materials. Therefore, nanomaterials have high microwave absorption characteristics. As shown in Fig. 3.2, the size, shape, charge, and delivery method of nanomaterials are beneficial to improve the permeability and bioavailability of drugs. What's more, after these performance optimizations and upgrades, the ability of the material to penetrate the BBB can be further improved, and its therapeutic effect is better than traditional therapies.

3.3.1 Size

Size is considered to be a crucial design factor, which directly affects the absorption of nanomaterials by the brain. Different material sizes can lead to special biological phenomena, including circulatory half-life and vascular penetration. The smaller the size of nanomaterials, the easier it is to cross the BBB, which means that it is more suitable for targeting and drug delivery. In principle, nanomaterials are generally considered to be better than 100 nm [25]. Although NPs of smaller size are easier to transport through the BBB, they cause limitations in encapsulation efficiency, rapid drug release, and surface energy limitations during endocytosis. When the size of the nanomaterial is less than 6 nm, it is easily filtered by the kidney and excreted from the body [26]. Nanomaterials around 20 nm are considered the ideal size for NDDs. It satisfies two conditions at the same time, escaping renal excretion and penetrating the BBB [21].

3.3.2 Shape

As shown in Fig. 3.2b, the nanomaterials have different shapes. In terms of spatial structure, the existing nanomaterials can be divided into four categories, namely, three-dimensional, two-dimensional, one-dimensional, and zero-dimensional. This depends on the nanomaterials meeting the nanoscale requirements in several dimensions. For example, nanorods and nanotubes meet the nanoscale in two dimensions of space, and they belong to the category of one-dimensional nanomaterials. Similarly, nanoparticles meet nanoscale in three dimensions and are classified as zero-dimensional nanomaterials [21]. In addition, studies have found that the entry of nanomaterials into cells, the circulation time of the drug throughout the body, and the choice of blood-brain barrier permeability are also affected by the shape of the nanomaterials. Round nanomaterials have the advantage of being easy to prepare, but they also have some shortcomings. The curvature of the round material limits the tight bonding with endothelial cells to a certain extent. After comparison, it is found that because the rod-shaped structure has a larger contact area with the receptor on the cell membrane surface, it has a tighter and more reliable bond and is easier to be taken up by cells. In summary, the shape of nanomaterials determines the permeability of the material to a certain extent. It is expected that improving the efficacy of therapeutic drugs and extending the cycle time can start from upgrading the shape of nanomaterials.

3.3.3 Charge

Because the cell membrane is negatively charged, it is generally believed that it is relatively easier for cells to take up nanomaterials with neutral and positive charges rather than negative charges. However, since the negatively charged nanomaterials

have less binding to the proteins in the blood, the blood circulation time of the negatively charged materials is prolonged. In addition, under the premise of not destroying the integrity of the blood-brain barrier, negatively charged nanomaterials have strong permeability. Therefore, combining the advantages and disadvantages of these three types of materials, more negatively charged or neutral nanomaterials are selected in the application of NDD.

3.3.4 Delivery Methods

In order to overcome the selective permeability, improve the delivery efficiency, and improve the clinical effect, many explorations have been conducted on the drug delivery route of nano-drugs. Among them, intravenous administration is the most common way of administration. Through this method of administration, the nanomedicine can enter any vascular tissue including the brain. However, this method also has some problems that need to be solved urgently. How to avoid the rapid elimination of drugs from the body to prolong the time of systemic circulation and how to avoid accidental accumulation of drugs in nontarget organs, and thus more accumulation in target tissues and organs, are two problems that cannot be avoided by intravenous administration. In fact, the development of some new transportation methods has become a new research direction for scientists. Their view is that ultrasound-assisted nano-drug delivery has the potential to overcome the problems caused by these traditional delivery methods.

In summary, nanomaterials have the advantages of adding imaging agents, penetrating the vascular barrier, preventing drug degradation, and prolonging the time of drug in the systemic circulation. These have created more possibilities for the application of nanomaterials in NDD.

3.4 NDD

NDD is a complex degenerative disease of the CNS. It is characterized by a large number of irreversible loss of specific neurons, which eventually leads to chronic progressive disability or even death [27, 28]. Neurodegeneration is the gradual loss of neuronal structure and function, including neuronal death and glial cell balance, which can lead to cognitive impairment such as dementia [1, 29–31]. High glutamate concentration in the intercellular space can cause toxicity to neurons, resulting in aging and death of neurons. The excitotoxicity of glutamate is closely related to the occurrence and development of various NDDs. Nonspecific immunity is also called innate immunity. It protects the body through rapid response to various harmful substances. However, harmful substances also cause damage by activating the nonspecific immune, and such stimulation cannot be controlled [32, 34]. Mitochondria are important intracellular calcium stores. The exchange of calcium ions between the endoplasmic reticulum and mitochondria has a profound impact on

cell fate. Failure to clear free radicals in time leads to an imbalance between the body's oxidation and antioxidants. Under these stimuli, the endoplasmic reticulum releases its stored calcium ions, and then the mitochondria take up calcium ions, causing calcium overload and causing damage to the mitochondria. Mitochondrial damage will lead to the release of cytochrome c, triggering the formation of apoptosome, which activates Caspase-9, which in turn activates Caspase-3, the direct executor of apoptosis, resulting in neuronal cell apoptosis [32, 33]. Mitochondria are the power plants of most eukaryotes and also the only organelles containing DNA. Eighty to ninety percent of the energy required by cells comes from mitochondrial oxidative phosphorylation. The structure of mitochondria is divided into outer membrane, interstitial space, inner membrane, and matrix from outside to inside. The normal potential gradient between the structures is the basis for maintaining the normal function of mitochondria. Neurons can only obtain energy through aerobic metabolism of glucose consumption, and mitochondrial dysfunction will make neurons lack energy. Because of the complexity of brain function, the treatment of such diseases has been a difficult problem. With the development of nanomaterials and nanotechnology, it is believed that nanomaterials can penetrate the blood-brain barrier, assist in the diagnosis or treatment of NDD, and even participate in the improvement of patients' motor symptoms and the regulation of nervous system functions. The following part will specifically illustrate the advantages and applications of nanomaterials from several typical NDDs.

3.4.1 AD

AD is considered to be one of the most common NDDs, which is an irreversible and progressive disease of the nervous system [35, 36]. At present, there is no effective way to improve symptoms and cure diseases. AD is characterized by the accumulation of proteins in the tangles and plaques of nerve fibers, the death of neurons and glial cells, and the impairment of cognitive function caused by aging or genetic mutations. In these disease states, tangles and plaque aggregates or other stimuli can lead to chronic inflammation. This neuroinflammation leads to the death and progression of disease of cells such as neurons, astrocytes, and oligodendrocytes. The main clinical symptoms of AD are learning and cognitive impairment and dementia [37–39]. In late stages of AD, patients will experience irritability, confusion, and behavioral changes [40, 41]. Amyloid plaques and neurofibrillary tangles (NFTs) are characteristic substances in the brain of AD patients [42–44]. The formation of amyloid plaques is due to the weakened metabolism of amyloid precursor proteins, and eventually the accumulation of these plaques leads to nerve damage [45]. Under physiological conditions, NFTs are pairs of helical filaments that support neuronal growth-related tau proteins. However, in the case of excessive phosphorylation, they are toxic to cells. The diffusible ligands and oligomers in toxic amyloid (Aβ) plaques are important factors that directly lead to neurotoxicity. The location and number of Aβ plaque deposition are directly related to the diagnosis of dementia and the number of neurons [27, 32–34, 46]. In the past, scientists believe that the

breakthrough point in AD treatment lies in how to inhibit Aβ deposition and how to clear the already produced Aβ [47]. Based on the research on the mechanism of neurodegeneration, the concept of nerve cell protection has been put forward. There are three ways to protect nerve cells from degenerative changes: promoters that inhibit degenerative changes in nerve cells (such as microglia, nitric oxide), blocking the signal transduction process of degenerative changes of nerve cells, and activation of endogenous neuroprotective mechanisms (such as neurotrophic factors). With the deepening and complication of nanotechnology research in recent years, more possibilities for nanomaterials have been discovered and proposed, including the diagnosis, prevention, and treatment of AD [48, 49]. These nanomaterials, ranging in size from 6 to 100 nm, have significant advantages in preventing kidney excretion and crossing BBB. In addition, they also reduced the immune rejection of the host and greatly improved the biological safety of the material. Some nanomaterials realize their functions through specific binding with Aβ. In general, nanomaterials can be used as carriers to carry drugs across the BBB or as antioxidants and anti-apoptotic drugs to treat or prevent AD.

Tetrahedral DNA nanostructures (TDNs) are formed by four different DNAs (DNA, Table 3.1) based on the principle of basic complementary pairing (Fig. 3.3) [50–57]. In the past, it was found that drugs with neuroprotective or therapeutic effects on AD are harmful to nerve cells to a certain extent. From this aspect, TDNs have advantages in biosecurity and biocompatibility. What's more, small size, special structure effect, and resistance to nuclease are also advantages of TDN [2, 56, 58–68]. Via upregulating ERK1/2 phosphorylation and activating ERK1/2 signaling pathway, TDNs have the potential to protect PC12 cells from Aβ25-35-induced apoptosis [57]. CCK-8 assay, flow cytometry, Western blot, real-time fluorescence quantitative PCR, immunofluorescence, and other techniques were used to verify that the nanomaterial can promote cell proliferation, inhibit apoptosis, restore nuclear morphology, and reduce intracellular reactive oxygen levels. Zhang and colleagues used polylactic acid-glycolic acid copolymer to deliver basic fibroblast growth factor to target Aβ oligomers in the brain. After specifically binding with Aβ, this nanomaterial assisted in the removal of Aβ [69]. Other nanomaterials including polyethylene glycol-AU and reconstituted high-density lipoprotein (rHDL) have similar effects as PEG-PLGA [47]. These nanomaterials with neuroprotective effects on AD have good biological safety and can cross the BBB.

The nanocomposite, called NC-KLVFF, is wrapped with protein molecules that contain a cross-linked Aβ-binding peptide (KLVFF) polymer layer prepared by in situ polymerization [2]. NC-KLVFF is able to significantly improve the morphology of Aβ polymer by forming nano-clusters. The nontoxic Aβ/NC-KLVFF complex, formed by NC-KLVFF and Aβ, can impact the aggregation of neurotoxic Aβ, leading to reduction of neurotoxicity of Aβ. Due to the decrease of Aβ oligomers, the inflammation and neuronal damage were also alleviated, and this had been proved in vivo. There are some other kinds of nanomaterials, including gold nanorod, apolipoprotein E3-reconstituted high-density lipoprotein ApoE3-rHDL, graphene oxide nanosheets, and poly(n-butylcyanoacrylate), which are also recognized as candidate for AD therapy and make an effect in three ways: (1) changing the

Table 3.1 Sequences of ssDNA

ssDNA	Direction	Base sequence
S1	5′→3′	ATTTATCACCCGCCATAGTAGACGTATCACCAGGCAGTTGAGACGAACATTCCTAAGTCTGAA
S2	5′→3′	ACATGCGAGGGTCCAATACCGACGATTACAGCTTGCTACACGATTCAGACTTAGGAATGTTCG
S3	5′→3′	ACTACTATGGCGGGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGCCCATCC
S4	5′→3′	ACGGTATTGGACCCTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATGCTCTTCCCG

Fig. 3.3 Sketch map of TDNs

morphology of Aβ oligomers and reducing the toxicity, (2) carrying drugs through the BBB and reaching the lesion, and (3) forming the sandwich structure with Aβ oligomers which is easier to be removed.

Regarding the application of nanomaterials in the diagnosis of NDD, it has been proposed that nanoscale diagnostic methods are very useful in detecting early Aβ oligomers. Nanoprobe is composed of magnetic nanostructure of MRI and Aβ oligomer antibody. After penetrating the BBB, it can effectively and selectively bind to the target. Finally, the detailed information of Aβ oligomers, including the location, size, and structure, can be detected by imaging techniques and further help to diagnose AD. Mirkin et al. applied nanomaterials as a DNA carrier to investigate the concentration of Aβ oligomers in the early cerebrospinal fluid (CSF) of AD patients. In brief, CSF was exposed to both gold nanoparticles to DNA-functionalized Aβ oligomer antibodies and magnetic nanoparticles bounding to Aβ oligomer antibodies. Subsequently, two kinds of antibodies specifically bind to Aβ oligomers, and a special sandwich complex used for Aβ oligomer detection was developed.

3.4.2 PD

The pathogenesis of PD is due to abnormal basal ganglia function, resulting in abnormal accumulation of Lewy bodies in the substantia nigra and the reduction of dopaminergic neurons [70–72]. PD, a motor disorder that eventually progresses to cognitive dissonance, also has an age and genetic basis, and protein aggregation is more complex than that of AD. Although most PD is idiopathic PD, some patients have known genetic mutations that complicate the search for new therapies. The exact cause of PD is unclear. It is currently believed that the degeneration and death of PD dopaminergic neurons can be related to genetic factors, environmental factors, oxidative stress, and aging [73–76]. Patients with PD will first experience tremor or awkward movements on one limb, which will further affect the contralateral limb. As the disease progresses, patients will experience clinical manifestations including static tremor, bradykinesia, stiffness, and postural gait disorders. Currently, drugs (levodopa preparation) are mainly used clinically as the main treatment for PD, but they can only slightly improve the symptoms and cannot prevent the progression of the disease [77]. Inhibition of neuronal apoptosis and abnormalities of α-synuclein

are recognized as key to the treatment [72]. More and more nanomaterials have been designed for the diagnosis, prevention, and treatment of PD in recent years. These new nanomaterials can be used as drugs themselves or as carriers to carry drugs effectively into the brain through the blood-brain barrier, inhibit neuronal apoptosis, and reduce the accumulation of Lewy bodies, thereby preventing motor dysfunction and preventing the deterioration of the disease when compared with traditional drugs. In addition, magnetic nanomaterials can be used as an auxiliary means of MRI to diagnose PD early.

Tang et al. proposed a novel drug delivery system with few side effects, which improved the therapeutic effect [78]. They designed a nanoparticle to coat dopamine which was modified with borneol and lactoferrin (Lf-BNPs) and prepared by double emulsion solvent evaporation. This nanomaterial can promote the absorption of dopamine by SH-SY5Y, and it has low toxicity to cells after double modification. In addition, intranasal administration has been shown to effectively reduce striatum damage caused by 6-hydroxydopamine. Gan and his team designed a nanoparticle that has been shown to reduce pro-inflammatory cytokines and activate the B-cell pathway. This nanomaterial is prepared by coupling rabies virus glycoprotein (RVG29) [79]. Based on the effect of Zn on amyloid formation, Girigoswami used the human erythromycin amyloid model to compare the anti-amyloid capacity of ZnO nanoparticles (ZnO-NP) and ZnO nanoflowers (ZnO-NF) [80]. They designed a series of experiments and proved that ZnO-NF is more suitable for PC12 cell amyloid degradation than ZnO-NP due to the effect of surface ratio. In previous studies, deep brain stimulation (DBS) was considered an effective way to deal with PD. On this basis, many scientists are committed to applying nanomaterials and metal particles in this therapy. Xiao and colleagues modified the sensitive microelectrode with a nanocomposite of reduced graphene oxide and platinum nanoparticles (Pt/rGO) [81]. Microelectrode arrays (MEA) were used to monitor changes in dopamine concentration in real time after applying this modified nanomaterial to brain damage in PD animal models. Human-induced pluripotent stem cell (iPSC) transplantation has neuroprotective and repairing effects on PD. When combined with cyclosporin A (CsA), it can reduce the rejection of the host and improve the survival of iPSC. Yu et al. designed a nanoparticle of polylactic acid and glycolic acid containing cyclosporin and transplanted this nanocomposite together with iPSCs into the striatum of 6-hydroxydopamine-injured rats [82]. The measurement results of human marker Stem121 and the immunoreactivity of tyrosine hydroxylase (TH) indicated that the immune rejection of iPSC was greatly reduced after a month. Ahlawat et al. prepared a chitosan nano-molecule using particle gelation. This nanomaterial was subsequently demonstrated to have antioxidant and anti-apoptotic properties in the cell model of rotenone-induced PD [83].

The aforementioned new nanomaterials provide new possibilities for the application of nanomaterials in NDD, such as nanotechnology detection and partial discharge therapy. Nanoparticles have the advantages of size, shape, and charge, which can make them pass through BBB effectively. Combining these nanomaterials with certain traditional materials not only increases the ability of traditional drugs to penetrate the blood-brain barrier but also reduces host immune

rejection caused by the new materials, achieving a win-win situation. In the next step, we will continue to explore the destination of metabolites of these new nanomaterials. Because whether they degrade or accumulate in the brain will adversely affect neurons.

3.4.3 HD

George Huntington first clearly described HD as an inherited NDDs [84]. It is a fatal neurodegenerative disorder characterized by mental, cognitive, and motor impairments. From the initial chromosomal localization to the detection of the Huntington protein gene, the genetic analysis of HD has been the leading study of hereditary neurological diseases. Studies have shown that an increase of more than 34 in CAG repeats leads to seizures in affected individuals. The mutant Huntington protein can accumulate, negatively affect mitochondrial function and metabolism, and inhibit the expression of brain-derived neurotrophic factor (BDNF) and other nutritional factors. Behavior disorder (delayed acquisition of motor skills), unconscious movement (dance disorder), and cognitive impairment (dementia) are the three major characteristics of HD [85]. Patients with HD were healthy and showed no signs before entering the symptoms. However, as the disease progresses and worsens, the patient may die due to serious complications such as inanition, fall, aspiration, restlessness, and difficulty swallowing [85]. The prevalence of HD was about 2.71 per 100,000 once reported in a systematic review [86]. The pathophysiological feature of HD is the abnormal CAG repeat amplification at the 5' end of the Huntington (Htt) gene (including extended polyglutamine extension). The gene is located at p 16.3 on chromosome 4, which causes the accumulation of abnormal unstable proteins [87]. The mutated Huntington protein can denature cells by altering the metabolism of neurons, causing damage to the striatum of the brain [88]. The severity of HD is closely related to the degree of repetition of polyglutamine sequence. Although the current research has not fully explained the mechanism of selective degeneration, it is generally believed to be closely related to inflammation, metabolic disorders, transcriptional disorders, and proteolytic changes [89].

Despite decades of progress made by clinicians, the treatment of HD still stops at improving symptoms. Tetrabenazine (TBZ), the only drug approved by the FDA to treat HD chorea, can inhibit vesicular monoamine transporter 2 (VMAT-2) [90]. However, when TBZ improves the symptoms of HD, it also brings serious side effects. Studies have shown that some participants who received TBZ treatment exhibited depression and suicidal behavior. Once use of TBZ is stopped, it may worsen chorea [91, 92]. In addition, there is currently no effective treatment that can improve cognitive symptoms. Depression is the most common psychotic symptom in HD. How to control refractory depression well is still a challenge.

The presence of mutant Htt protein is an important factor in the pathogenesis of HD, and silencing the expression of mutant Htt is considered to be one of the

potential treatment methods for HD. Godinho synthesized a nanoparticle modified with β-cyclodextrin to send siRNA to the CNS. The study found that the expression level of mutant Htt protein in the experimental group using the nanomaterial was significantly reduced [93]. Debnath designed a new type of nanomaterial to successfully prevent the aggregation of mutant glutamine-containing Htt protein in neuronal cells. It consists of an ionic polymer shell of trehalose wrapped with an iron oxide core, which can reduce protein fibrillation in the extracellular space [94]. Peptide inhibitors (QBP1-QBP6, NT17, PGQ9P2, PGQ9P2,3, PGQ9P1,2,3, and P42) can inhibit abnormally aggregated polyglutamine and are therefore considered as one of the potential treatments for HD. In addition, Joshi and his colleagues verified the function of poly-D,L-lactide-co-glycolide nanoparticles loaded with PGQ9P2, QBP1, and NT17 in two classic cell models of HD and a *Drosophila* model [95]. Some researches designed some new kind of nanomaterials to eliminate the abnormal accumulation of Htt, which contains metal particles. Zhang found that MnFe2O4 nanoparticles can degrade mutant Htt protein through ubiquitin-proteasome system and reverse cell death in vitro. This material is coated with dextran as a surfactant to synthesize $MnFe_2O_4$ nanoparticles [96]. Ceccon designed nanomaterials capable of removing Htt protein targeting Met7 oxidation in the httNT domain, which is called TiO_2 nanoparticle, and its surface has a catalytic oxidation effect [97].

Such nanomaterials containing metal particles do have the potential to inhibit or eliminate the accumulation of misfolded proteins. However, the solution of some problems needs more in-depth exploration. Specifically, the functions of some nanomaterials have only been verified in cells, and their ability to penetrate BBB and their effects on brain function need to be further verified by in vivo experiments. How to avoid the accumulation of metal particles and metabolites of the nanomaterials in the brain after the nanomaterials played their role, which affects safety, will be a difficult problem to be solved in the next step.

Solid lipid nanoparticles (SLNs) are colloidal carriers that can deliver hydrophobic drugs to the CNS. The advantages of using SLNs include no unpleasant odor or taste, the ability to cross BBB, and reduced dosage for efficient delivery [98]. Mitochondrial dysfunction is one of the important factors leading to HD, so a lot of research has focused on restoring mitochondrial function. Curcumin with anti-inflammatory and antioxidant functions was encapsulated in solid lipid nanoparticles (CSLN), and a new delivery system was established. The system was proved to be effective in treatment of HD animal model [98]. Thymoquinone (TQ), the main biologically active phytochemical component, is derived from the seeds of *Nigella sativa*. TQ has been shown to have the potential to be applied for many diseases. Ramachandran et al. wrapped TQ with SLNs and applied it to 3-NP-induced HD mouse model. It can be observed that the muscle strength and memory of HD mice after treatment are improved through the hook activity, space navigation task, forced swimming test, and string test [99]. They found that TQ-SLN can reduce NMDA receptor sensitization and resist neuroinflammation to relieve movement disorders. At the same time, it can also inhibit the activation of microglia [100]. Bhatt and his team synthesized SLN loaded with rosmarinic acid. The behavioral assessment of

HD rats was significantly improved, when delivering the nanocomposite to the brain of Wistar rats by nasal administration [101].

Previous studies have found that cholesterol synthesis levels in HD mouse models are significantly reduced [102]. Thus, it is considered that cholesterol is one of the potential therapeutic targets of HD treatment due to the important role of cholesterol in physiologic function of cells. However, due to the effect of fat solubility, cholesterol cannot cross the BBB. Scientists thought that nanoparticles, wrapping cholesterol, can be new to overcome this difficulty. Valenza et al. designed a nanoparticle rich in cholesterol. After being injected into HD mice, the nanoparticles can significantly improve the cognitive function of mice [103]. Furthermore, Belletti et al. designed a nanoparticle called MIX-NPs, which can efficiently load cholesterol and can be absorbed by neuronal cells and release cholesterol in neuronal cells [104].

Current research supports the view that different treatments for HD are required at different stages. Thus, it is obvious that early diagnosis is particularly important throughout the whole treatment process. The combination of magnetic nanoparticles and imaging technology was introduced into the early diagnosis applications of HD. Liu and his team synthesized a nontoxic and PEGylated superparamagnetic oxidized nanoparticle (SPION), which contains amyloid oligomeric scFv antibody (W20). After intravenous injection of this composite material into the HD mouse model, it successfully provided signals for different focus areas and healthy areas [105]. Although the mechanism is unclear, numerous studies have illustrated a point that bone marrow mesenchymal stem cells (MSCs) have the potential to be used in the treatment of NDD [89]. In order to explore the specific mechanism, Moraes and his team proposed using SPION to label MSC. After applying SPIONs-MSCs to the HD rat model, it was observed that the behavior of mice was significantly improved and neurogenic brain damage was alleviated. The nanoparticles have great prospect in both treatment and diagnosis of HD. SPIONs-MSCs were detected in the HD lesion, shown by the results of MRI [106].

3.4.4 Other

Generally speaking, cerebral infarction (CI) is a cerebral tissue infarction caused by cerebral artery occlusion [107]. Huang et al. believed that embolization can be targeted by covalently binding magnetic nanoparticles in polyacrylic acid and tissue plasmin activator. In their research, they found that nanocomposites can reduce the area of CI mouse embolism caused by iron oxide and accelerate thrombolysis [108]. Mei and colleagues have designed a nanocomposite material that has been shown to inhibit the expansion of brain damage and reduce the area of cerebral infarction after brain injury. This material encapsulates tissue plasmid activators in self-assembled antioxidant nanoparticles [109]. So and colleagues first used cerebral artery occlusion (MCAO) to induce the construction of CI animal model and then encapsulated the acid salt in liposomes (LITA) and injected it into experimental

animals [110]. MRI-assisted examination found that LITA reduced anterolateral ventricular (ALVv) [110]. The current research results show that that nanomaterials can play a role in assisting diagnosis and treatment, for example, imaging agents or carriers of small molecule drugs. However, it cannot be ignored that the metabolite of these nanomaterials is a problem of fate. This is also a problem to be solved in the research on nanomaterials in the future.

3.5 Conclusion

In conclusion, this review summarizes the latest applications and application prospects of nanomaterials in NDD. In the current research, some new nanomaterials have been developed as anti-apoptosis, antioxidant, and anti-inflammatory agents for NDD treatment. This type of material protects the vitality and function of nerve cells through anti-inflammatory and antioxidant properties and thus participates in the treatment of NDD. The accepted view is that the misfolding and abnormal aggregation of certain proteins lead to the occurrence of NDD. The original purpose of designing such nanomaterials was to treat NDD by inhibiting protein misfolding, reducing aggregation, or promoting clearance. In addition, some magnetic nanomaterials that can pass through the blood-brain barrier are good imaging agents and have the potential to detect and diagnose NDD with the help of MRI. In addition to the application and improvement of the ability to penetrate the blood-brain barrier, the application of nanomaterials in degenerative diseases is mainly focused on avoiding the accumulation of drugs in nontarget organs and host immune rejection. Although nanomaterials currently exhibit amazing advantages and potential in NDD applications, there are still some problems that need to be further explored and resolved. For example, it is not yet known whether the metabolites of nanomaterials will form polymers and accumulate in the brain, causing uncontrollable adverse consequences. At the same time, it is unknown whether these polymers are more likely to be intercepted by the BBB due to changes in the space organization. These problems require scientists to invest more time and energy to solve them, especially in the constantly evolving field of nanomaterials.

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Chapter 4
Application of Nano Drug Delivery Systems in Inhibition of Tumors and Cancer Stem Cells

Dexuan Xiao and Ronghui Zhou

Abstract For its high mortality rate, cancer has posed a significant threat to human's lives. Every year, more than 3.4 million people died for cancer all over the world. The main therapeutic methods for cancer include surgery, chemotherapy, and radiotherapy. However, surgery is only conducted for patients with early-stage cancers; chemotherapy and radiotherapy have obvious side effects. In addition, many researches have indicated that cancer stem cells play a crucial role in tumor recurrence and multidrug resistance. Compared with traditional drug carriers, nano drug delivery systems have many advantages in targeting delivery, combination therapy, etc. In recent years, more and more nano drug systems are applied in clinical practice, and various multifunctional nano drug systems are designed to kill cancer stem cells. Our review introduced the main problems in anticancer therapy for cancer stem cells, and the developments of several nano drug delivery systems.

Keywords Cancer · Nano drug delivery systems · Cancer stem cells · Chemotherapy · Targeting therapy · Combination therapy

4.1 Introduction

Today, cancer has produced a great threat to human's lives for its highest mortality rate. Tumor cells tend to metastasize to healthy organs and then cause invasion and end in multiple organ failure to death. Every year, 3.4 million patients die of cancers all over the world [1]. The main treatments for cancers include surgery, chemotherapy, and radiotherapy. Nevertheless, surgery is conducted for patients with early-stage cancer, so metastasized tumors cannot take surgery. Chemotherapy and radiotherapy have toxic side effects, which usually cause serious damage to patients' immune and hematopoietic systems. The combination therapy becomes

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common treatment for malignant tumors. Different drugs unite to exert synergistic effects that can improve anticancer activity and reduce toxic side effect [2]. Nevertheless, drugs usually have different pharmacokinetic properties due to their physicochemical properties, resulting in different distribution ratios in vivo, so in the combination therapy seldom achieve the prospective results. In addition, many researches have reported that cancer stem cells play a crucial role in tumor recurrence and multidrug resistance.

In the past decades, nano drug delivery systems have a dramatic development, some of which has been used in clinical practice. Compared with traditional drug carriers, nano carriers show various advantages, such as better stability, targeting, biocompatibility, etc. In our review, we mainly summarized the advances of nano drug carriers in inhibiting cancer and its stem cells in recent years.

4.2 Cancer Stem Cell

Recent studies have indicated that cancer is difficult to cure because tumor tissues are made up by heterogeneous cell populations, in which there are not only rapidly proliferating tumor cells but also a little number of cancer stem cells (CSCs) with stem cell nature [3]. In 1997, Bonnet et al. [4] first proved the presence of CSCs in a patient with acute myeloid leukemia. Since then, a variety of CSCs has been successfully isolated and cultured [5–8]. The theory of CSCs believes that tumors are heterogeneous cell population. There is a part of cancer cells similar to embryonic stem cells, which can unlimitedly self-renew and divide and regulate the occurrence and development of tumors [5, 9, 10]. Conventional chemoradiotherapy mainly targets ordinary tumor cells, while CSCs are not sensitive to this. On the other hand, with the stimulation of radiotherapy and chemotherapy and the elimination of ordinary tumor cells, the microenvironment for CSCs changes. CSCs are enriched, and their capabilities of proliferation, invasion, and metastasis can be further enhanced. They may show more durable resistance to chemotherapy drugs. Some studies indicate that implanting a small number of CSCs into mice could reshape the phenotype of the tumors, which show that CSCs often lead to tumor recurrence, drug resistance, and metastasis [10, 11].

Just like other ordinary stem cells, CSCs highly express various stem genes, such as OCT4, NANOG, and SOX2, as well as stem signal pathways, such as Wnt/β-catenin, PI3K/Akt, NF-κB, PTEN, and JAK/STAT [12–14], to maintain the stemness of CSCs and regulate the development of tumors. The biological characteristics of CSCs determine that CSCs are different from ordinary tumor cells. CSCs are usually hidden in cancer cells and in a resting state. CSCs can weaken the effect of drug-induced DNA damage, enhance the ability to repair DNA damage, and maintain the stable genetic inheritance. These biological properties of CSCs are controlled by complex intracellular and extracellular regulatory networks.

4.2.1 Cell Cycle Arrest

CSCs are dormant and proliferate inactively in most cases. Many chemotherapeutic drugs tend to target dividing cancer cells. Ordinary tumor cells usually stay in the G2 or S phase, while CSCs tend to stay in the G0/G1 phase, so CSCs react insensitively to chemotherapeutic drugs. Due to the effect of radiotherapy and chemotherapy, ordinary tumor cells are eliminated, and CSCs can be enriched. At the same time, for the stimulating effect of chemotherapeutic drugs, stationary CSCs is quickly activated and enter the G2/S phase to proliferate and divide, resulting in tumor recurrence. Cioffi et al. [15] found that the expression of cyclin-dependent protein kinase inhibitor P21 and tumor suppressor P53 was increased in drug-resistant pancreatic tumors, while the expression of cyclin D1 was decreased, and cells were arrested to the G0/G1 stage. As a result, tumor cells were not sensitive to common chemotherapeutic drugs, and they often led to tumor recurrence. Acetaldehyde dehydrogenase 1 (ALDH1) is often considered as a sorting marker for CSCs. It can oxidize aldehydes to carboxylic acids, resist the damage of alkylating agents, and is highly expressed on the surface of CSCs. Meng et al. [16] found that the sensitivity of CSCs with positive ALDH1 expression was insensitive to chemotherapeutic drugs. ALDH1A regulated cell cycle by regulating KLF4 and P21, and CSCs could rest at the G0/G1 phase. In addition, glycogen synthase kinase-3 (GSK-3) promoted ubiquitination of β-catenin through Wnt/β-catenin signal pathway, blocked the activation of downstream target cyclins-1 and c-Myc, and inhibited the progress of cell cycle.

4.2.2 Drug Efflux

Chemotherapeutic drugs require specific drug concentration to exert their killing effect. Compared with ordinary tumor cells, the concentration of drug is much lower inside CSCs, which is related to high expression of multidrug resistance (MDR) on the surface of CSCs. The ABC transporter family is a type of MDR, namely, ATP-binding cassette protein. It can exert the energy released by ATP hydrolysis to exclude the therapeutic drug out of cells, resulting in low drug concentration inside cells, which finally leads to drug resistance and tumor recurrence. In addition, ABC transporters can also be used as tumor prognostic factors. ABC subfamily C member 2 (ABCC2), ABCC3, and ABCG2 are markers of CSCs, and their expression is related to the prognosis of patients with colon cancer. Because a drug can be excreted by multiple transporters, inhibiting a specific transporter alone cannot hinder the efflux of the drug. Wu et al. [17] found that the tyrosinase inhibitor, tepotinib, could inhibit ABCB1-mediated drug efflux, but could not block the efflux effect of ABCG2 and ABCC1.

4.2.3 DNA Damage Tolerance and DNA Damage Repair

Nowadays, parts of chemotherapeutic drugs kill tumor cells by inducing damage to tumor cell DNA. CSCs are not only in a resting state in most cases but also have a strong resistance to DNA damage and the ability to repair DNA damage. The main mechanism is that damaged DNA, on the one hand, can be removed by excision repair. On the other hand, CSCs with damaged DNA can enhance recombinational repair. Cisplatin inhibits DNA replication and transcription of tumor cells and induces tumor cells to apoptosis. Srivastava et al. [18] found that the highly expressed DNA polymerase Polη in ovarian CSCs could avoid the damage of cisplatin by skipping the damaged DNA replication point to promote DNA synthesis. In addition, DNA damage repair can be accomplished by enhancing the repair of DNA double-strand break. Gold et al. [19] found that the antitumor mechanism of spironolactone was inhibiting the repair of DNA double-strand break of tumor cells. Because spironolactone had no effect on normal CSCs, it only inhibited the DNA damage repair of CSCs where DNA double-strand break occurred. Spironolactone weakened the DNA damage tolerance of CSCs and inhibited the DNA damage repair. It could interfere with CSCs division, resulting in eliminating CSCs and inhibiting tumor recurrence and drug resistance.

4.2.4 Epithelial Mesenchymal Transformation (EMT)

EMT means that epithelial cells abandon the characteristics of epithelial cells and express the nature of interstitial cells and get the ability to invade and metastasize. EMT plays a crucial role in the generation of CSCs and is related to biological characteristics of CSCs and drug resistance. Currently, it is believed that parts of CSCs are generated by dedifferentiation of differentiated tumor cells. Some studies [20] have indicated that the upregulation of EMT transcription factors, such as snail and slug, could induce differentiated tumor cells to dedifferentiate into CSCs and produce chemical resistance. Shuang et al. [21] found that the expression of stem genes was enhanced in the tumor cells with EMT, indicating that the occurrence of EMT could dedifferentiate tumor cells into CSCs and obtain stem characteristics. Meanwhile, CSCs overexpress mesenchymal markers. Wang et al. [22] found that while pancreatic cancer cells highly expressed the stem markers, like CD44 and NANOG, the expression of EMT transcription factors, such as snail, also increased. Gao et al. [23] found that in liver CSCs, $CD44^+$ CSCs highly expressed mesenchymal cell markers, like vimentin and N-cadherin, but lowly expressed epithelial cell markers. Removal of $CD44^+$ CSCs could inhibit the ERK/Snail signaling pathway to weaken the metastasis of liver cancer cells. Many evidences show the direct connection between EMT and CSCs [24, 25]. EMT and CSCs can jointly promote tumor invasion and metastasis and regulate the occurrence and development of tumors. EMT is a dynamic and reversible process. Therefore, by directly targeting one or several EMT-related transcription factors, it is not possible to inhibit the occurrence of EMT.

4.2.5 Tumor Microenvironment

The drug resistance of CSCs is not only related to their stem characteristics but also regulated by tumor microenvironment. CSCs alone cannot survive and require the support of tumor microenvironment. Tumor microenvironment includes not only tumor cells themselves but also tumor-related stromal cells, microvessels, interstitial cells, and cytokines. Tumor microenvironment is dynamically changing and regulated by tumor development. Hypoxia, low pH, and low glucose supply of tumor microenvironment further maintain the stem characteristics of CSCs and improve drug resistance. CSCs in the dormant state can adapt to low energy supply in the microenvironment. Once the environment changes, the energy utilization mode of CSCs will change accordingly and enter the proliferation stage [26]. Therefore, tumor microenvironment of CSCs is an important factor in maintaining stem characteristics, driving tumor development, recurrence, and drug resistance. The same tumor has significant differences in gene expression and biological behavior in different tumor microenvironments, which may be related to the differences among tumor microenvironments.

4.3 Nano Drug Delivery Systems

According to clinicopathological and physiological studies, there are obvious differences in the structure of normal cells and tumor cells. Tumor tissues are characterized by poor vascular integrity due to the porous structure of capillaries (the pore size is 100–780 nm [27]). Furthermore, due to the collapse of lymphatic vessel wall and the loss of lymph circulation in tumor tissues, macromolecules and lipid particles cannot be absorbed back into blood through lymphatic system, so macromolecules and lipid particles are easily taken up and retained by tumor tissues, making them easily play the corresponding biological effect in tumor tissues [28]. This effect is named the enhanced permeability and retention effect (EPR) [29, 30]. Nano drug carriers make use of this effect of tumors and prepare drug delivery systems in the nano-size category. The passive targeting of nanomedicine is based on the EPR, which makes blood circulation time increased in specific tumor tissues, thereby enabling nano drugs to selectively concentrate in tumor tissue and perform better therapeutic effects [31]. Nano drug delivery systems can be roughly divided into liposomes, polymeric micelles, and other nanoparticles. Compared with traditional drugs, nano drug delivery systems have more advantages.

4.3.1 Strengthen Drug Stability

There are various enzymes in the human body, which can destroy and degrade drugs in the process of drug absorption. As a result, drugs are easily lost during blood circulation. Nano drug delivery systems can encapsulate drugs and provide protective effect by external physical barrier, which significantly strengthen the stability of drugs. In the process of implanting nano drug delivery systems into body, not only the loss of encapsulant in blood circulation is avoided, but also the dose dependence of traditional drugs can be changed, which directly improves drug efficacy.

4.3.2 Enhance Drug Targeting

Nano drug delivery systems can change the distribution of drugs to a certain extent. Nano drug carriers can enhance drug targeting and reduce toxicity by decreasing drug leakage to other healthy tissues [32]. During the design process of nano drug carriers, surficial materials can be assigned reasonably and modified according to specific physical and chemical properties, to change drug load, pharmacokinetics, and biocompatibility. At the same time, the targeting of nanoparticles to cells or molecules can be further enhanced, leading to the sustained release and better stability. The occurrence and development of tumors are very rapid. Tumors cannot form integral blood vessel wall; thus, a large number of pores will be formed. The diameter of these pores is nanometer-scale, so that nano drug delivery systems can reach the diseased tissues and organs.

4.3.3 Better Degradability

Due to their large specific surface, small particle size and strong adsorption capacity, nano drug carriers degrade more completely than traditional carriers. Nano carriers can increase the binding time of drugs to the affected part and further increase the absorption rate of drugs. According to pharmacokinetics, if drugs cannot be effectively degraded, there is a risk of toxic effects.

4.3.4 Increase Bioavailability of Drugs

Nano drug carriers can enhance the permeability of encapsulant to biofilms. Nano drug carriers enable drugs pass through blood-brain barrier or biofilm more efficiently, thereby improving the bioavailability of drugs. Many oral macromolecule drugs are hard to take effects for the first pass elimination. Nano drug carriers can

improve the solubility of macromolecular drugs that are difficultly absorbed by oral administration, resulting in higher concentrations of drugs in tumor tissues and better drug utilization and treatment effects.

4.4 Liposomes

Alec Bangham first built the hollow phospholipid structure, which laid the foundation for the liposomal model in 1965 [33, 34]. After that, many phospholipid bilayer structures were designed [35]. Gregory Gregoriadis put forward the idea that liposomes might perform well in drug delivery systems [36, 37]. A part of articles suggested that liposomes had influence on the distribution of encapsulant in vivo [38–40]. On the other hand, some researchers utilized liposomes to deliver the chemotherapeutic agent—cytosine arabinoside—and significantly increased the lifetime of mice bearing L-1210 leukemia [41, 42]. It was the first time for liposomes to enhance the activities of wrapped drugs. Other small molecular therapeutics entrapped in liposomes were also in the attempt and showed improved better effects for animal disease models [43–46].

Liposomes are hollow vesicles encased in the lipid bilayer, whose diameters range from nanometers to a few micrometers. Due to their good biocompatibility, easy modification, and specific targeting, liposomes have been generally applied in fields of drug carrier [47]. Phospholipid and cholesterol are the main compositions of liposomes. The most common phospholipid used in the liposome includes phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid. The cell membrane is also composed of phospholipid, so liposomes have good biocompatibility and low toxicity. Phospholipid is an amphiphilic molecule, which include nonpolar skeleton and polar head. According to phospholipid's charge, four categories of liposomes are classified, including uncharged liposomes, positively charged liposomes, negatively charged liposomes, and zwitterionic liposomes. The charge of liposomes significantly determines the liposomal property [48]. The most common liposomes are positively charged, and these liposomes tend to attract cell membranes based on electrostatic interaction, leading to an increase in cellular intake of carriers. In addition, the positively charged head assists to achieve lysosomal escape based on "proton sponge effect" and reduce the degradation of drugs in lysosomes. Positively charged liposomes are suitable carriers for nucleotide therapeutics, because DNA or RNA is also negatively charged [49]. Another main component for liposomes is cholesterol. About 30 percent composition of cell membrane is cholesterol, and it is usually neutrally charged. Cholesterol plays a key role in the properties of liposomes. The interaction between fatty acid chain of phospholipid and cholesterol contributes to maintaining the stability of liposomes [50, 51]. Furthermore, cholesterol can control the rigidity of bilayer structures [52] and condense phospholipid molecules to enhance the density [53]. It brings about a more ordered structure in the tail area, along with low polarity [54], increased bilayer viscosity, and enhanced

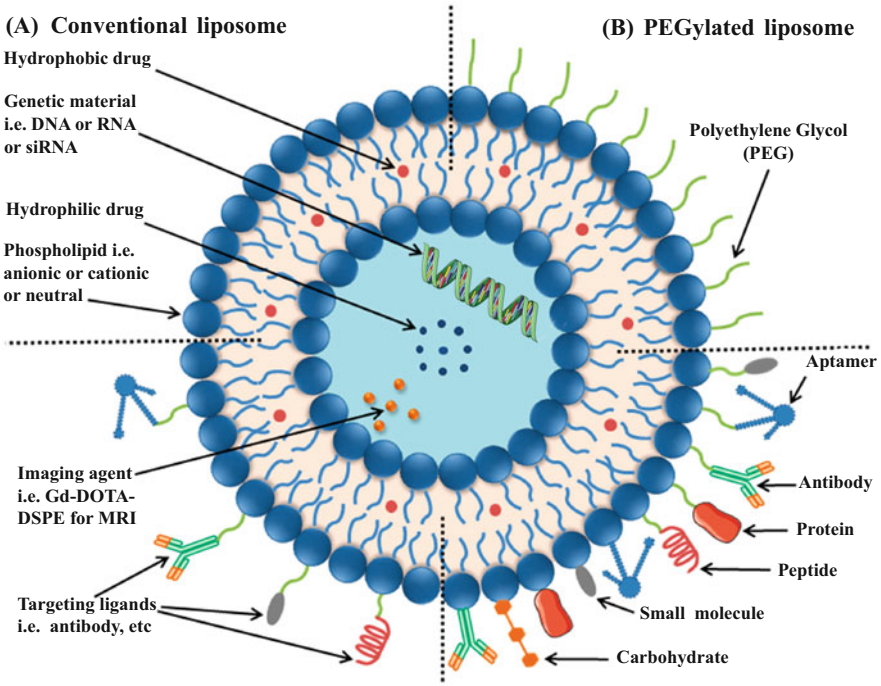

Fig. 4.1 The development of liposomes in different stages. (**a**) Convention liposomes; (**b**) PEGylated liposomes; (**c**) ligand-targeted liposomes modified with aptamer, antibody, protein, peptide, etc.; (**d**) multifunctional liposomes. Copied with permission [57]. Copyright 2018, International Journal of Molecular Sciences

membrane rigidity [55]. In addition, cholesterol is sure to affect liposome size, heighten permeability, and modulate the releasing of encased drug [56]. Finally, to overcome the disadvantages of liposomal carriers, various functional agents are applied to decorate on the surface of liposomes, such as antibodies, polyethylene glycols (PEGs), aptamers, ligands, proteins, peptides, and some other small molecules (Fig. 4.1).

Liposomal drug carriers can efficiently transport drugs to targeting tumor and heighten the accumulation of drugs, so they have great application potential and clinical value [58]. Liposomal preparations are usually administered by intravenous injection. When circulating in the vascular system, liposomes get nonselective adsorption with serum proteins and tend to be eliminated by macrophages, resulting in low targeting. Although lots of liposomes have shown positive results in vitro experiments, they hardly survive in the complex in vivo environment. To make liposomes more effective in vivo, the function of liposomes undergoes further optimization in various ways.

4.4.1 Overcoming the Quick Elimination by MPS

The conventional liposomes were found to get rapid elimination from circulation. As liposomes interact with negatively charged serum proteins, the mononuclear phagocyte system (MPS) works [59]. As a result, liposomes might accumulate in organs, mainly including spleens or livers, leading to the rapid decrease in the blood concentration of entrapped drugs [60, 61]. Except for the treatment of MPS diseases, the phenomenon significantly weakens the targeting of liposomes to their targeted tumors and produces toxicity to the MPS organs [62, 63].

Considering these problems, a large number of liposomes without entrapped drugs were injected into bodies ahead of time. This method was to shield the MPS and prolong the circulation time of the liposomes with drugs [64]. Juliano et al. [65] did some research on the blood circulation of the liposomes labeled with Tc. The results indicated that the radiation intensity of tumors in the group with MPS shielded was 1.5 times higher than that in the unshielded group; at the same time, tumors reflected two-time radioactivity of other tissues. Nevertheless, it was impossible to conduct this shielding method in clinical practice. Hence, modification of liposomal properties to increase the circulation time in vivo had become the focus of researches. Some early studies showed that reducing vesicle size, to a certain extent, could prolong the circulation half-lives in vivo [66]. A possible mechanism for the rapid elimination of liposomes was that those serum proteins had an influence on them and the surficial modifications of liposomes caused the increase in the circulation half-lives. In early studies, the focal point was on the differences between the unmodified phospholipid bilayers and the biological bilayers whose facial membranes were abundant in carbohydrates. The monosialoglyprotein GM1 was first added in the liposomes imparted by egg phosphatidylcholine (egg PC), leading to realizing the increased circulation time without MPS shielding [67]. It suggested less MPS intake and longer circulation half-lives of liposomes by substituting sphingomyelin for egg PC. People speculated that it might be for an increase in the facial hydrophilicity of these long-circulating liposomes composed of carbohydrates.

Some scholars found that the circulation half-lives of liposomes were increased when they added polyethylene glycol (PEG) as composition [68]. It was milestone progression for Maruyama et al. [69] and Cevc et al. [70] to modify PEG molecules on the surface of liposomes. The PEG could envelop liposomes and separate liposomes for serum protein. In this way, the PEGylated liposomes extremely weakened the quick elimination by MPS and made improvements in the circulation half-lives in vivo [71, 72]. Additionally, Ji et al. [73] indicated that the PEGylated neutral liposomes (NL) were more instable than cationic liposomes (CL) and anionic liposomes (AL) and NL loaded with DOX was inferior to CL and AL in antitumor activity.

However, the PEGylated liposomes are also faced with problems. First, if the PEGylated shell is not opportunely removed from tumor tissues, the uptake of the liposomes into the cancer cells may be inhibited, or it is hard to achieve lysosomal

escape [74]. In addition, Dams et al. [75] found that the PEGylated liposomes were observed to arouse accelerated blood clearance (the ABC phenomenon), which appeared at the first conduct of the PEGylated liposomes by intravenous injection. When injecting the PEGylated liposomes repeatedly at intervals, their pharmacokinetics should abnormally change. The occurrence of this phenomenon not only weakened the long-circulating advantages of the PEGylated liposomes but also caused serious damage to healthy organs or tissues.

4.4.2 Constructing Active Targeting Liposomes

The active targeting liposomes are constructed based on the interaction between ligands and receptors to realize specific active targeting. However, specific receptors have saturation effect, and these liposomes modified with ligands are weak in active targeting. In addition, complex microenvironment in tumor area tends to hinder them from approaching the targeting receptors [76, 77]. To solve these problems and promote the targeting efficacy, three methods are taken into consideration. First is to construct liposomes with dual-targeting molecules. Second is to utilize physical factors to realize active targeting. Third is to apply cell-penetrating peptide technology in liposomes.

To improve the targeting efficiency and accuracy, liposomes can be modified with two kinds of ligands [78]. More and more attentions come to dual-targeting liposomes. Li et al. [79] designed targeting liposomes modified with folate and transferrin for DOX delivery. Transferrin guided liposomes to penetrate through blood-brain barrier and then approached brain tumor. On the other hand, folate could also target the glioma cells and release the active pharmaceutical ingredients that made DOX effective. Furthermore, by inhibiting the ATP-binding cassette transporter, transferrin could decrease drug efflux and restrict drug resistance. The results indicated that compared with single-modified liposomes, dual-modified liposomes did better in active targeting without obvious DOX toxicity to heart.

Physical chemistry targeting liposomes mean application of some physicochemical methods to enable targeting agents effective in specific regions, such as pH-sensitive liposomes, photoactive liposomes [80]. By application of physical targeting technology in liposomes, liposomes could remain stable in complex microenvironment and accumulate in targeting tumor. Yu et al. [81] designed a novel liposome by modification with folate and the near-infrared imaging agent, naphthalocyanine green (IR780). The liposome featured with photoactivity and loaded DOX to kill cancer cells. The results indicated that the liposome significantly enhanced the targeting to liver tumor and the release of the entrapped drug could be under control. By diffusion to tumor tissues, the entrapped drug could extremely restrict the microcirculation of liver tumor.

Cell-penetrating peptides (CPPs) are segments of short positively charged peptides. By electrostatic interaction, CPPs can approach cell membrane and assist drugs enter cells without toxic effects. CPPs are lack of cell selectivity, and blood

enzymes tend to degrade CPPs in blood circulation. The application of CPPs in liposomes is expected to enhance the permeability of liposomes and promote the drug accumulation in tumor area [82].

Plenty of experiments have been conducted to make out what advantages the active targeting liposomes possess compared with the passive targeting ones and which would have the practical applying value. Several articles reported the improvements of active targeting liposomes in survival periods compared to passive targeting ones [83, 84], while no improvements in other cases [85, 86]. The passive and active targeting liposomes approached the target tissues in the same distribution method. Therefore, if they have similar circulation half-lives in vivo, active targeting liposomes will have no advantages in distributing to tumor tissues [87, 88]. More liposomes absorbed by target cells rather than target tissues seem to achieve improvements in the survival period. If entrapped agent releases ahead of intake, the anticancer effect will be hard to improve.

4.4.3 Realizing Triggered Release of Drug

Conventional liposomes release entrapped drugs by passive diffusion. Although the modification of PEG on the surface of liposomes contributes to more circulation time and higher targeting efficiency, more circulation time means liposomes have more opportunities to gradually release the active pharmaceutical ingredients ahead of time. Moreover, drug release is also influenced by serum proteins [89, 90]. Cholesterol is benefit to improve the stability of bilayers and reduced drug leakage from liposomes [91, 92]. It was also helpful to reduce the release of drugs in advance when the liposome membrane switched from the liquid phase to the solid phase [93]. The more stable bilayer exactly decreased the drug release ahead of time, but this might reduce the efficiency of passive diffusion in targeting area, which would lead to drug resistance.

To solve these problems, scholars are trying to construct novel liposomes to realize the triggered release of drug. Two triggers are mostly utilized—local triggers (such as enzymes and pH changes) and remote triggers (such as light, ultrasound, and heat).

Mangy attentions come to tumor hyperthermia in recent years. Hyperthermia is a feasible treatment for terminal cancers or tiny tumors. Compared with operation, hyperthermia takes less expenditure and seldom damages adjacent tissues. The heating source mainly includes ultrasound, laser, microwave, and radio. The heating source could raise temperature up to 50 °C. The active enzymes could be denatured, and transient cytotoxicity could be produced at such temperature [94]. At the same time, when thermosensitive materials applied, heat is expected to trigger the drug release. Chen et al. [95, 96] constructed a thermosensitive liposome by addition of ammonium bicarbonate. When the temperature raised to 42 °C, ammonium bicarbonate tended to degrade and produce bubbles, leading to the crash of bilayer structure and the release of entrapped drug.

Under light irradiation, some photosensitizer can release singlet oxygen and damage cancer cells, which is termed as photodynamic therapy. Skin cancer, oral cancer, and cervical cancer are expected to be cured under photodynamic therapy [97]. Moreover, light irradiation is able to trigger the drug release. There are some unsaturated bonds in the structure of liposomal bilayer, and singlet oxygen can break these unsaturated bonds, leading to the destruction of hydrophobic chains and the triggered release of drug [98].

Another method for triggered release is using enzyme-responsive liposomes. Some enzymes are abundant in tumor tissues, such as secreted phospholipase A2 (sPLA2), matrix metalloproteinases (MMPs), prostate-specific antigen (PSA), urokinase plasminogen activator (uPA), and elastase. These enzymes are expected to be triggers for drug release [99]. Li et al. [100] reported a MMP-2 reactive liposome with β-cyclodextrin modified. When approaching tumor area, under the function of MMP-2, the liposome tended to separate into two parts. One part was β-cyclodextrin, an anti-fibrotic drug; the other part was the liposome with RGD modified. The liposome showed stronger lethal effect to pancreatic cancer.

The release rate of drug plays a role in liposome function. If entrapped drug cannot release from carriers, carriers have no value. In addition, the release mode and rate should be taken into consideration, too.

4.4.4 Constructing Multifunctional Liposomes

In order to kill cancer cells, different chemotherapeutics tend to inhibit different signaling pathways. Nevertheless, one chemotherapeutic conducted repeatedly might cause drug resistance [101]. In addition, therapeutic effect of one drug is hard to achieve expected results. The common method to make up for it is increasing the dose, but the following toxicity is also a problem. Hence, to solve the problems of one-drug treatment, doctors tend to conduct combination therapy. Combination therapy is uniting two or more chemotherapeutics with complementary effects, which is expected to minimize side effects and inhibit drug resistance [102].

The uniting of several chemotherapeutics does benefit to therapeutic effect. Liposomes can load chemotherapeutics with similar function, resulting in less drug dose and improved anticancer effect. DOX is an anthracycline antibiotic and able to treat multiple cancers, such as lymphoma, lymphoma, and breast cancer [103]. DOX mainly takes function by destroying chromosome [104]. First, utilizing electrostatic action, DOX is able to intervene into DNA double helix. Next, DOX can inhibit the DNA-topoisomerase II and stop DNA double helix to rewind, leading to the end of the cell replication process. Third, DOX leads cells to apoptosis. Cisplatin is a conventional alkylating agent. Cisplatin leads to DNA break and cell apoptosis by terminating DNA synthesis or transcription [105]. Ramasamy et al. [106] reported a transferrin-modified liposome to deliver cisplatin and DOX. The results indicated that the liposome entrapping cisplatin and DOX got better anticancer effect than complex of cisplatin and DOX, let alone other one-drug formulations. Salinomycin

(SAL) is a carboxylic acid polyether antibiotic that can effectively inhibit the growth of tumor stem cells, but its poor water solubility limits its application. The combination of SAL and other chemotherapeutics has dramatically improved the therapeutic effect on tumors. Gong et al. [107] prepared three liposomes respectively loaded with SAL, DOX, SAL, and DOX (SLN, DLN, SDLN). The results indicated that SDLN could more effectively inhibit the growth of lung cancer than SLN and DLN in vivo and the number of CSCs in the tumor site was significantly reduced. They also found that SDLN had the best synergistic effect when the drug ratio SAL/DOX was 1:1. Therefore, exploring the optimal ratio of two drugs is a key issue in the preparation of dual drug-loaded nanoparticles.

Liposomes can deliver chemotherapeutics with different functions to take synergistic effect. Paclitaxel (PTX) is a natural extract and widely applied to treat multiple cancers, such as breast cancer, ovarian cancer, gastric cancer [108], non-small cell lung cancer and Kaposi's sarcoma [109]. This agent functions uniquely. At cell mitotic phase, PTX can lead tubulin proteins to polymerize and make the microtubules dysfunctional, eventually resulting in cell death. However, the anticancer effect of PTX is weakened for its low water solubility. Scientists tried to deliver PTX with liposomes, which significantly increased the water solubility of PTX and reduced side effects, including vomiting, nausea, and hypersensitivity reactions, compared to free PTX [108]. What's more, Fang et al. [110] designed a multilamellar liposome to deliver PTX and DOX. These two drugs function based on different mechanisms. The results suggested that after the treatment with dual agent liposomes, the survival period of tumor-bearing mice was much longer than that only treated with single drugs.

Gene transfection technology is introducing normal genes or genes with therapeutic effects into relative cells to cure diseases aroused by gene disorder. This type of treatment is called gene therapy. Scholars found that genetic changes played a crucial role in the development of tumors. The expression of miRNA and siRNA in tumor cells, especially in CSCs, is often abnormal, which affects the self-renewal and reproduction of cells. Therefore, miRNA or siRNA can be used as a therapeutic to inhibit cancer cells [111]. In addition, tumor suppressor genes can suppress tumor cells and CSCs by regulating the expression of specific enzymes. However, nucleic acid drugs have many disadvantages, such as low cell uptake, poor stability, and bad tissue specificity. Liposomes can effectively deliver nucleic acid drugs for their specific properties. MiRNA200c is significantly downregulated in breast CSCs, and increasing its content can restore the sensitivity of PTX. Liu et al. [112] selected CL to transport miRNA200c by charge attraction. After breast CSCs ingested the therapeutic for 12 h, miRNA200c could still be effectively released from the liposome, and breast CSCs became more sensitive to PTX. Kim et al. [113] designed a liposome to deliver small-molecule drug and modified a single-chain antibody against transferrin receptor on its surface. Using this nanocarrier to load wtp53, it was able to pass through the blood-brain barrier, downregulate methylguanine methyltransferase (MGMT) by wtp53, and weaken drug resistance of malignant glioma cells and CSCs to temozolomide (TMZ).

Nowadays, multidrug resistance has become the main obstacle to the chemotherapy of tumors. Nowadays, varieties of anticancer agents are combined to overcome drug resistance by nano delivery systems. The agents should be chemotherapy sensitizers or inhibit tumor cells from agent efflux. Cyclosporine A and verapamil are included [114]. However, these inhibitors of efflux pump have toxicity on healthy organs or tissues. Calcium channel inhibitors, such as verapamil, might induce hypertension, dizziness, and arrhythmia, while cyclosporine A often leads to immunosuppression, nephrotoxicity, and leukopenia [115–117]. These problems extremely restricted the application of efflux pump inhibitors. Resveratrol, a natural extract, attracts more focus on its functions in recent years. Some studies indicated that resveratrol took effects on anti-inflammatory, anti-aging, anti-oxidation, and reducing blood sugar [118]. Resveratrol was also reported to suppress tumor proliferation and induce cell apoptosis [119]. Guo et al. [120] constructed a PEGylated liposome to deliver resveratrol and PTX. The results were positive; the nano delivery system could effectively kill breast cancer cells and significantly restrict the development of tumors in mouse model without obvious toxicity.

4.5 Polymeric Micelles

In 1992, Yokoyama et al. [121] first reported polymeric micelles as nano drug delivery systems after the research of DOX-conjugated block copolymer micelles. Polymeric micelles have special core-shell structures that are self-assembled by amphiphilic polymers in aqueous phase. The particle size of polymeric micelles is generally less than 200 nm, and they have different shapes, such as balls, bars, and tubes, among which the balls are the most common ones. The hydrophilic shell of polymeric micelles can maintain their spatial stability and long circulation. The hydrophobic core can encapsulate hydrophobic drugs to increase water solubility of drugs. Like other nano drug delivery systems, polymeric micelles can approach tumors through the EPR. In addition, modification of ligands or antigens on the surface of polymeric micelles can achieve active targeting.

The chemical properties and molecular weight of the hydrophilic shell significantly affect the stability, pharmacokinetic, and tissue accumulation of polymeric micelles. The most common hydrophilic shell polymers are polyethylene glycol (PEG), polyethylene oxide (PEO), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), and chitosan. The hydrophilic shell sometimes is made up of a mixture of various polymers. These hydrophilic polymers give stealth capabilities to polymeric micelles, allowing them to avoid absorption by the reticuloendothelial system (RES), which is significant to achieve long circulation time in vivo. PEG is nontoxic, non-immunogenic polymer and has good water solubility. PEG can effectively avoid the interaction with immunoglobulin, prevent polymeric micelles from uptake by phagocytes, and increase the circulation time in vivo [122]. N-succinyl chitosan is a derivative of chitosan. Compared with chitosan, N-succinyl chitosan is more

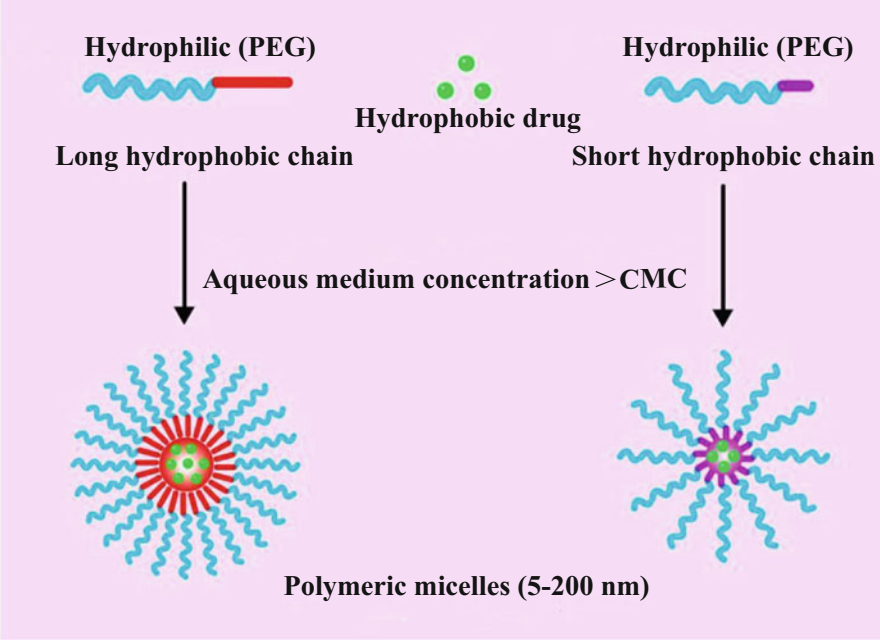

Fig. 4.2 General structure of polymeric micelles loaded with hydrophobic drug. Copied with permission [127]. Copyright 2019, Frontiers in Pharmacology

biocompatible and less toxic, has a longer half-life period, and can be effectively enriched in tumor sites. Thus, it is widely used in antitumor carries [123].

The hydrophobic core of polymeric micelles can be loaded with poorly soluble drugs to improve drug stability and bioavailability. The properties of hydrophobic core directly affect the stability, drug loading capacity, and drug release performance of polymeric micelles. Commonly used hydrophobic core polymers are polyesters and polyamino acids. Polyesters are easy to hydrolyze and have low toxicity and good biocompatibility. Polyesters mainly include polylactic acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), and polylactide-co-glycolic acid (PLGA). Polyamino acids can be used as core fragments. They are easy to modify chemically and encapsulate drugs in physical or chemical methods. Polyamino acids mainly include poly-L-aspartic acid (PAsp), poly-L-histidine (PHis), poly-L-glutamic acid, and their derivatives. In addition, poly-β-amino ester (PbAE) and some short-chain hydrophobic lipids, such as distearoylphosphatidylethanolamine (DSPE), have also been widely used as hydrophobic core polymers in recent years [124, 125]. Moreover, if the hydrophobic core is more similar to the chemical structure of drugs, the solubilization effect of the polymeric micelles on drugs will be better, and the solubility of drugs will be higher. Yokoyama et al. [126] substituted the benzyloxy group in the hydrophobic core of PEG-PBLA micelles with hexadecyl esters. As a result, the solubility of the aliphatic anticancer drug KRN-5500 was significantly increased (Fig. 4.2).

Polymeric micelles have good thermodynamic and kinetic stability. The thermodynamic stability of polymeric micelles is affected by a variety of factors, such as the interaction between the shell polymers, the interaction between the hydrophobic drugs and the core, the length of the hydrophobic block copolymers, the hydrophobicity of the core, the length and density of the hydrophilic block copolymers, and the liquid environment [128]. Therefore, in order to ensure the good thermodynamic stability of polymeric micelles, the length of the hydrophobic and hydrophilic blocks and the surface density of the hydrophilic blocks need to be balanced. The CAC value is an essential indicator for measuring the thermodynamic stability of polymeric micelles. When the copolymer concentration is above CAC, the micelles can be stable at the thermodynamic level. The CAC value of polymeric micelles is usually in the range of 10^{-7} to 10^{-6} mol L^{-1}, which is lower than that of micelles formed with low-molecular-weight surfactants (10^{-4} to 10^{-3} mol L^{-1}) [125]. Therefore, polymeric micelles can maintain structural integrity after a series of dilutions and have good thermodynamic stability. When the concentration of the copolymers drops below the CAC value, the kinetic stability of polymeric micelles becomes more important. The kinetic stability of micelles is mainly related to the structure of the micelle core, the size of the hydrophobic blocks, and the ratio of the hydrophobic blocks to the hydrophilic blocks. Unlike micelles formed by low-molecular-weight surfactants, polymeric micelles have a more stable hydrophobic core structure and better kinetic stability. Therefore, when below the CAC value, the decomposition rate of polymeric micelles will slow down, which can maintain the structural integrity of polymeric micelles before reaching targeting site, prevent drug leakage, and effectively improve drug bioavailability.

Compared with traditional drug delivery carriers, polymeric micelles have many potential advantages, such as broader drug delivery range, stronger delivery capacity, longer circulation time, fewer side effects, and better antitumor efficacy, so they have been widely used in anticancer therapy.

4.5.1 Passive Targeting and Active Targeting Polymeric Micelles

Polymeric micelles can deliver drugs to tumor tissues through the EPR, enabling passive targeting of nanocarriers. Theoretically, the circulation time of polymeric micelles in vivo is one of the most critical factors of the distribution of micelles in tumor tissues. Therefore, the micelles with long circulation time can take more substantial EPR effect, and the accumulation of micelles in tumor tissue can be more. However, because the EPR of different tumors is significantly different, the EPR alone to deliver polymeric micelles to tumor tissue is not ideal [129]. The tumor-specific active targeting nanocarriers mainly utilize specific molecules expressed on the surface of tumor cells or rely on the tumor microenvironment to load the modification of target molecules to achieve active targeting.

One method is the application of monoclonal antibodies that specifically bind to tumor antigens. As an ideal tumor antigen, monoclonal antibodies are expressed on the surface of tumor cells and necessary for tumor cells to survive. In addition, they are not prone to mutation [130]. Monoclonal antibodies can be independently used as targeting carriers for antitumor drugs or can be coupled to drug delivery systems or form complexes with drugs. Torchilin et al. [131] bound anti-myosin antibodies to polyethylene glycol-phosphatidylethanolamine (PEG-PE) polymeric micelles in order to target lung cancer cells. Comparing the PEG-PE micelles, the drug-loaded micelles, and the antibody-modified micelles, it was found that all three micelles were stable in vivo. However, the active targeting of the monoclonal antibody-modified micelles was significantly higher than others. The antibody-modified micelles could effectively deliver drugs to early tumors, mature vascular tumors, and metastatic lung tumors.

Another method is receptor-mediated targeting. Receptors on the surface of tumor tissues or cells are closely related to the growth and proliferation of tumors, and some are specifically overexpressed. The receptors can induce internalization of tumor cells after binding to the corresponding ligand, which helps to kill tumor cells. These receptors can be used as specific targets for tumor targeting therapies. At present, the common receptor types are cytokine receptors, transferrin receptors, low-density lipoprotein receptors, hormone receptors, and folate receptors [132]. It is worth noting that folate is a small class of nonimmune molecules, which is nonirritating to body and binds explicitly to folate receptors. Many studies have confirmed that folate receptors are overexpressed on the surface of various cancer cells, including breast cancer, ovarian cancer, brain cancer, kidney cancer, lung cancer, and bone cancer cells, which is not expressed on normal cells [133]. Yoo et al. [134] first designed a PLGA-b-PEG copolymer and then connected DOX to the PLGA end to form a DOX-PLGA-PEG complex; on the other hand, they bound folate to the PEG end. The results showed that folate-modified polymer micelles were more toxic than free DOX and in vivo experiments also confirmed that folate-modified polymer micelles could deliver more micelles to tumor tissues. Abou-ElNaga et al. [135] connected folate to the surface of PTX-loaded PLGA micelle, which could greatly increase the sensitivity of PTX to ovarian CSCs in vivo and reduce the expression of drug resistance genes ABCG2 and MDR1.

The third method is tumor-activated drug. These drug delivery systems rely on inactive complexes to interact with tumor microenvironment or specific molecules on the cell surface, thereby activating the complexes and releasing drugs. These drug carriers can increase drug concentration in tumor tissues and kill tumor cells more effectively.

4.5.2 Drug Co-delivery Systems

The combination of two or more different therapies may produce synergistic effects, which is a promising strategy. The combination therapy can improve therapeutic

effect, reduce side effect, and even reach multiple targets at the same time. Physical encapsulation, chemical linking, or both of them are usually applied in polymeric micelles serving as drug co-delivery systems.

Ke et al. [136] utilized a PEG-PUC/PEG-PAC mixed micelle system to physically encapsulate thioridazine (THZ) and DOX for inhibiting tumor cells and CSCs. The results indicated that the system has a higher loading capacity for THZ and DOX. Compared with mixture of free DOX and THZ, mixed polymeric micelles have stronger antitumor activity in vivo. Li et al. [137] used PLG-PLGA polymeric micelles to deliver SAL and docetaxel (DTX). The micelle could effectively kill gastric tumor cells and gastric CSCs, and its tumor suppressive effect in vivo was stronger than single nanocarrier and dual drugs.

The microenvironment of tumor is different from normal tissues. Because cancer cells need to synthesize fatty acids, nucleic acids, and amino acids continuously, the energy requirements are much higher. Therefore, inhibiting the energy metabolism pathway can also effectively inhibit tumor growth, making cancer cells more prone to apoptosis [138]. Krishannamurthy et al. [139] selected PEG-PUC and PEG-PAC copolymers to self-assemble into polymeric micelles. The inhibitors of energy metabolism pathways, phenformin (Phen) and gemcitabine (Gem), were loaded in the micelle through hydrogen bonds and ionic interactions. The results showed that in in vitro experiments, the combination of two drugs was more toxic to lung cancer cells and lung CSCs than a single one, which significantly inhibited tumor growth. At the same time, the micelle did not cause liver and kidney toxicity and had good biological safety.

Nowadays, the combination of drugs and genes has become a promising antitumor therapy, which has the advantages of overcoming drug resistance and improving gene transfection efficiency [140]. Polymeric micelles can form PIC micelles through electrostatic interaction with negatively charged genes. The surface charge on PIC micelles enables it to be modified by molecules with opposite charges, which provides a new method to construct multifunctional carriers. Zheng et al. [141] prepared PEG-PLL-PLLeu copolymers for co-delivery of DTX and siRNA. DTX is physically embedded in the hydrophobic core of PLLeu, and siRNA is electrostatically adsorbed to the carrier by PLL. Compared with single DTX or siRNA micelle system, it had better tumor suppressive effects.

4.5.3 Environmentally Responsive Polymeric Micelles

Environmentally responsive polymeric micelles have become research hotspots, which mainly include pH-responsive polymeric micelles and thermoresponsive polymeric micelles.

There is a pH gradient in human body. The pH of physiological environment in vivo is 7.4, while the pH of endosomes is 5.5–6.0 and that of lysosome is 4.5–5.0. The pH of normal tissues and organs is higher than that of tumor tissues. Polymeric micelles can be got uptake by target cells through receptor-mediated endocytosis.

After entering cells, polymeric micelles are enriched in endosomes and then enter lysosomes. Therefore, the pH gradient between normal tissues and tumor cells can be utilized to synthesize drug carriers, which greatly increase the bioavailability of drugs and achieve targeting delivery. PH-responsive polymeric micelles usually have pH-sensitive bonds such as amidine bonds, amino groups, acetals, or ketals. Osada et al. [140] reported a pH-responsive polycarbonate micelle for the controlled release of PTX. The pH-responsive micelle consisted of a PEG shell and a polycarbonate core containing acetal. The research indicated that the micelle was very stable at pH 7.4, but the acetal in the hydrophobic core could rapidly hydrolyze at pH 5.0, making the micellar swell, resulting in releasing PTX rapidly. Staurosporine (STS) is a common protein kinase inhibitor that can effectively kill CSCs. The combination of STS and other chemotherapeutic drugs can synergistically inhibit the growth of tumors. PEG-β-PAsp and epirubicin (Epi) are connected by a hydrazone bond to form a drug delivery polymeric micelle (Epi/m), and the hydrazone bond can be broken in acidic environment, leading to drug release. Kinoh et al. [142] encapsulated STS in Epi/m to form dual-drug delivery micelles (STS/Epi/m). After the micelle entered tumor cells, they were triggered by the acidic environment and simultaneously released two drugs, STS and Epi, which effectively inhibited the growth of CSCs. At the same time, by inhibiting the ABC transporter, the drug resistance of CSCs was weakened, and CSCs resistant to Epi were effectively eliminated.

Generally, the temperature of normal tissues is lower than that of tumor tissues. According to this characteristic, thermoresponsive polymeric micelles can be used as drug targeting deliver. When temperature changes, thermoresponsive micelles will undergo a phase transition from dissolution to insolubility. Typical thermoresponsive polymers include poly-N-isopropylacrylamide (PNIPAM), polypropylene oxide (PPO), etc. Peng et al. [143] successfully prepared thermoresponsive polymeric micelles, poly-NIPA-co-DMAEMA, through the radical polymerization. Under local heating, the micelles could slowly release drugs and significantly inhibit the growth of C26-derived colon cancer cells.

4.6 Conclusion

As a new type of anticancer drugs, nano drug delivery systems have excellent advantages in increasing drug targeting, improving drug bioavailability, and enhancing drug stability. CSC theory believes that CSCs are the root cause of tumorigenesis, drug resistance, and postoperative recurrence. Therefore, the eradication of CSCs is of great significance for cancer treatment. The nano drug carriers which target CSCs have broad prospects in tumor treatment, but their clinical application still faces many problems.

First of all, CSCs and normal stem cells share many signal pathways and surface markers. Therefore, the process of targeting CSCs may cause damage to normal stem cells. Second, CSCs are heterogeneous too. CSCs with different sources have

different surface markers. The heterogeneity of CSCs limits the efficiency of targeting CSCs in tumor treatment. Third, the binding efficiency of nano drug delivery systems and targeting molecules needs to be studied further, so as their stability after entering the systemic circulation. Last but not the least, the toxicity of nano drug carriers which target CSCs needs to be observed and explored for a long time. With the development of CSCs research and nano drug delivery systems, the combination of the two will be closer in the future and will provide a strong guarantee for cancer treatment.

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Chapter 5
The Application and Problems of Tetrahedral Framework Nucleic Acids as a Drug Carrier in Biomedicine Fields

Xiaolin Zhang and Yunfeng Lin

Abstract With the rapid development of DNA nanotechnology and the continuous improvement of DNA editing technology, various DNA nanostructures have been constructed. DNA is the carrier of biological genetic information, and almost all organisms contain DNA, so DNA nanostructure has good biocompatibility. Benefiting from its excellent biocompatibility and editable properties, this emerging material is showing promising applications in many fields. Tetrahedral framework nucleic acids (tFNAs) are one of the most widely used typical structures of DNA nanostructures. In recent years, DNA tetrahedral frame nucleic acids have become a focus of biomedical research because of its stable structure, nanometer size, excellent mechanical properties, convenient synthesis, and high yield. Besides, they have good biocompatibility and biodegradability and are rich in modification sites. Moreover, because they can cross cell membranes without any help, they have promising applications in building intelligent drug transport systems. In this paper, the development of DNA nanostructures, the application of DNA tetrahedral frame nucleic acid in drug delivery, and the current problems are reviewed.

Keywords DNA nanostructure · Tetrahedron frame nucleic acids · Drug carrier

5.1 Introduction

Of late years, DNA nanotechnology is advancing by leaps and bounds [1, 2]. In addition to the structural characteristics brought about by the nanoscale, such as surface effect, tunnel effect, small size effect, and so on, DNA nanostructures also possess the characteristics of good biocompatibility, editable property, and strong stability brought about by the nature of DNA structure. DNA nanotechnology has made remarkable progress since professor Seeman first prepared DNA

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nanostructures in 1982 [3]. DNA nanotechnology has come a long way since it was developed in the 1990s, from simple modular assembly to multiple origami structures. At present, various DNA nanostructures are applied in various fields such as molecular detection, tumor diagnosis, biomedicine, drug delivery, biomolecular assembly, biosensors, nanomolecular machines, targeted therapy, and so on [4, 5]. Tetrahedral framework nucleic acids (tFNAs) are a typical representative of DNA nanostructure, which is made up of four single strands of DNA that self-assemble. Tetrahedral framework nucleic acids have been widely studied because of its simple preparation method and high yield [6, 7]. Tetrahedral framework nucleic acid, due to its ability to enter cells, makes the biological imaging and intelligent drug transportation in cells and animals based on DNA nanotechnology become new development opportunities [8]. This paper reviews the latest research on DNA nanomaterials and introduces different functional modification methods of DNA tetrahedral nanomaterials, and the application of DNA tetrahedral frame nucleic acid in drug delivery and the problems faced are reviewed.

5.2 DNA Nanostructures

5.2.1 The Concept of DNA Nanostructures

DNA nanostructures are two-dimensional or three-dimensional nanomaterials composed of single strands of DNA following the principles of Watson-Crick base pairing. DNA molecule has remarkable molecular recognition performance and remarkable structural characteristics, which makes it have unique advantages in the nanoscale regulation of materials, and also shows a broad application prospect in many fields. DNA is stored in the nucleus as a vehicle for carrying genetic information, which is made up of four different deoxynucleotide molecules. It was Watson and Crick who first proposed in 1953 that DNA is a large molecule with a double helix structure [9]. Soon after, researchers have focused on DNA that can make accurate base complementary pairs and gradually applied it to fields such as medicine, genetics, and ecology. DNA is not only a vehicle for carrying genetic information of living organisms but also the ideal component of biological functional materials [10]. DNA nanostructures are composed mainly by the self-assembly of DNA molecules. Self-assembly is one of nature's main methods for assembling highly complex materials [11, 12]. DNA strand is assembled into a double helix structure with its complementary strand under the principle of exact base pairing by the synergistic action of hydrogen bond, stack, electrostatic, and hydrophobic. In the preparation of DNA nanostructures, the first step is to have a positive sequence design, and then the DNA molecules are spontaneously assembled into DNA nanostructures by intermolecular or intramolecular hybridization under appropriate solution conditions [13–15]. DNA self-assembles into DNA nanostructures following the principle of base complementary pairing. As a kind of nano-biological materials with precise structure and size, DNA nanostructures have wide application prospect in many fields.

5.2.2 *The Development of DNA Nanostructures*

With the advantages of bottom-up self-assembly strategy, high controllability, and precision, DNA nanotechnology based on DNA components has attracted the attention of related research fields. In 1982, professor Seeman put forwarded that DNA can form specific structure through Watson Crick's pairing principle and the structure of the single can form in sticky end complex two-dimensional or three-dimensional structure, leading the whole of the development in the field of DNA nanotechnology [16, 17]. After this, the researchers in DNA, for "building materials," by "bottom-up" constructing method, designed and synthesized DNA, and the DNA of different shapes of various functional nanostructures, DNA nanotechnology has penetrated into many research fields [18–20]. In the early days of DNA nanotechnology, the synthesized structure was only a cross and topological structure formed by a number of single DNA strands paired with complementary bases. In 1993, Seeman's team designed multi-crossing structures that guarantee the mechanical strength of DNA nanostructures, such as multiple crossing sites between the double helix domains that forming rigid planar structures [21]. After all these, a large number of three-dimensional polyhedron structures were synthesized by the method of multichain base pairing, such as tetrahedron. Then, Mao et al. made further improvements, greatly reducing the types of DNA strands needed, reducing costs and experimental errors [22]. Rothemund invented the DNA origami in 2006, and the emergence of the technology makes the production of complex DNA nanostructures ability improved greatly [23]. The DNA origami is a nucleation self-assembly process, and the whole process passes through several nucleation points at one time. Therefore, the complexity of self-assembly of graphic modules generated by DNA origami is greatly increased. Through DNA origami techniques, researchers have constructed a variety of intricate nanopatterns and nanostructures, including smiley faces, dolphins, maps of China, huge rocks, nuts, bridges, flask, stereoscopic vases, pentacle stars, squares, rectangles, triangles, hollow boxes, tetrahedrons, and cubes [24–26]. In addition, each staple chain in a template constructed by DNA module or DNA origami can be extended to a specific identifiable sequence, which allows templates constructed by DNA nanotechnology to be further functionalized and widely used in many research fields [27].

With the continuous progress of technology, DNA nanostructures begin to move from the purely basic, structural research to specific applications. In the 552 issue of Nature magazine in 2017, four articles were published in the form of cover articles, which consisted of the mass synthesis of single DNA strand, the construction of large-scale DNA structure by DNA nano-tiles, and the 2D DNA origami patterns made by combining computer programming [28–30]. These directions show us the wide application prospect of DNA nanostructure. As it turns out, DNA nanostructure is developing faster and applied in more directions than we thought. When it comes to applications of biological and medicine fields, DNA nanostructures are widely used in the design of biosensors [31–34]. Considering the chemical nature of DNA nanostructures, multiple oligonucleotide sequences or aptamers are connected to

various DNA nanostructures and constructed as biosensors of microRNA or protein, so as to be used in the detection of various diseases. DNA nanostructures also show promising prospects as drug carriers [35, 36]. Through the characteristics of easy editing of DNA, researchers modified drugs or aptamers on the DNA nanostructure to obtain the targeted drug delivery with good biocompatibility. For example, Kim et al. reported for the first time a DNA mirror nanostructure which is self-assembled, with good biocompatibility and greater serum stability [37]. On this basis, after incubating doxorubicin, Kim et al. obtained stronger tumor inhibition effect in vivo and in vitro than the conventional way of drug administration. DNA nanostructures have also made a breakthrough in the field of gene editing, a hot topic in recent years [38, 39]. For example, Gu et al. achieved efficient transfection of CRISPR-cas9 by using the structure of DNA strands, providing a new idea for gene editing. Beyond biomedicine, DNA nanostructures play a role beyond our wildest dreams [40]. Organick published an article in Nature Biotechnology in February 2018, claiming that the writing and reading of binary data in DNA has been realized by taking advantage of the easy editing of DNA and benefiting from the extremely high data density of DNA, which is expected to bring technological innovation in big data storage [41]. It is very clear that with the increasing understanding of DNA nanostructures, interdisciplinary research is beginning to make groundbreaking breakthroughs [42]. These researches and advances have shown us a new material and structure motif with broad application prospect, and it is exerting broad and far-reaching influence in related fields.

5.3 Tetrahedral Framework Nucleic Acids

tFNA is one of the most widely studied DNA nanostructures in recent years. DNA tetrahedral nanomaterials are simple and strong pyramidal 3D structural models, which have high mechanical rigidity and stability, rich in functional modification sites, simple production process, and high yield [43–46]. In particular, the DNA tetrahedral nanomaterials based on synthetic models, such as Turberfield, have shown promising applications in biological detection, in vivo imaging, gene carrier, and drug delivery [47]. DNA tetrahedral framework nucleic acids have some unique advantages over traditional DNA nanostructures in terms of biocompatibility, relative stability, and ease of editing.

5.3.1 Self-assembly

tFNAs are three-dimensional DNA nanostructures with tetrahedral shape, which is formed by the automatic hybridization of each strand through the clever DNA sequence design and the principle of complementary pairing [48–50]. Each ssDNA are divided into three segments, the three pieces respectively with other

three different pieces ssDNA hybrid tetrahedral structure formation, and each small piece of the two hybrid combinations form tetrahedron has a double helix structure. On the tetrahedron vertex, ssDNA 5′- and 3′- at the end of each intersection, forming a port or on the edge [51]. DNA materials can achieve fine control of material molecules at the nanoscale. All at once, making the two adjacent sides of DNA tetrahedron at a certain angle, so as to ensure the correct formation of the structure of DNA tetrahedron and a certain degree of stability, each single strand of DNA between the adjacent two small fragments contains one base or two which are not paired with other sequences [52, 53]. Furthermore, in order to synthesize a regular tetrahedron of DNA, each ssDNA fragment must contain the same number of bases [54–56]. According to the Watson-Crick complementary base pairing principle, the four ss DNA were added to the TM buffer solution in equal quantities, and the four single-stranded DNA could be automatically complementary hybridized into a tetrahedral three-dimensional DNA structure through a one-step annealing operation. The synthesis conditions are as follows: 10 min at 95 °C, after that cooling to 4 °C for 20 min [57–60].

5.3.2 The Physical, Chemical, and Biological Characteristics

5.3.2.1 The Nanoscale and Editable

The DNA molecule is 2 nm wide; the distance between the two bases is 0.34 nm, and as a result the double helix structure increases by about 3.4 nm per 10 base pairs (bp) [61]. These properties determine the size adaptability of nucleic acid molecules. The size of DNA molecule determines that DNA material is an ideal nanomaterial. tFNA is a three-dimensional tetrahedral structure composed by multiple single-stranded DNA through the principle of complementary pairing of bases. The shape and size of tFNAs can be precisely controlled [62–64]. Researches show that the efficiency of the cells uptake of DNA nanostructure was influenced by its size and shape [65]. DNA nanostructures vary in size from nanometer to micron. This flexibility allows us to try a variety of sizes to compare and adjust the efficiency of endocytosis.

DNA molecules have a continuous length of 150 bp. When the double strand length is less than 150 bp, the DNA strand is rigid and not easily bent. According to this characteristic of DNA molecule, its rigidity and flexibility can be controlled by changing the number of bases on the double helix strand. Using this characteristic of DNA materials, researchers can design different rigid and flexible control nanomaterials according to different needs [66]. At present, the length of tFNAs used in most studies is 21 bp, which is rigid.

5.3.2.2 The Ability to Enter Cells

The efficiency of a material's uptake by cells is not only affected by its charge but also largely related to the geometric properties such as the morphology and size of the material [67, 68]. Many investigations have shown that nanomaterials with a scale of 10–100 nm are relatively easier to be actively absorbed by cells [69]. Recent research has shown that DNA nanostructures can freely enter cells with great efficiency. The cell membrane is the main barrier for DNA oligonucleotides entering into a cell. Since the cell membrane is negatively charged, common DNA single strand and double helix can hardly enter the cell through the membrane. While DNA nanostructures are negatively charged, their unique nanoscale properties allow them to enter cells through endocytosis (including endocytosis and pinocytosis), an energy-dependent active transport process rather than simple diffusion [70]. Fan Chunhai's team observed the interaction between RAW264.7 macrophages and fluorescently labeled tFNAs by confocal microscopy. After 2 h of culture, strong fluorescence signal was observed in the cytoplasm, which indicated that the DNA tetrahedral nanostructures can be ingested by cells. In the meantime, the single-stranded DNA that synthesis the tetrahedron was incubated with the cell, and only very weak fluorescence signals detected in the cytoplasm illustrated the formation of the tetrahedral structure for effective cells intake is important [71]. Flow cytometry instrument quantitative analysis results also show that compared with single DNA, after forming tetrahedral nanostructures, the amount taken up by cells increased significantly. Moreover, the tetrahedral structure of DNA can effectively resist the degradation of nucleases in biological culture medium and extracellular and inactivated fetal bovine serum. The tetrahedron structure remained intact after 4 hours of incubation. After the cells were incubated for 8 h, the cells and the DNA tetrahedron marked by Cy3 and Cy5 fluorescent markers are still well coincidently aligned, which fully proved that the formation of DNA nanostructures has a good stability. It is also can be used in biomedical research in the field of one of the important characteristics. Besides, Liang et al. found that, unlike the traditional DNA structure, tFNAs can cross the cell membrane to a certain extent and obtain a certain degree of lysosomal escape by modifying the nuclear nucleic acid aptamer [67]. This suggests that tFNAs may have a biological effect on cells that ordinary nucleic acids do not have, and at the same time, it may lead to breakthroughs in targeted drug delivery as a carrier and in biological imaging and intelligent drug transportation in animals. However, research on the movement of DNA nanostructures into cells and where they end up in cells is still in its infancy and controversial.

5.3.2.3 Biocompatibility and Biodegradability

DNA, as a natural biomolecule existing in all biological systems, has a large number of regulatory tool enzymes. Therefore, DNA nanomaterials have good biocompatibility and have no toxicity to organisms [72]. Moreover, DNA nanomaterials have

good biodegradability because they it can be degraded by a variety of DNA enzymes. For DNA nanostructures, the shape and size do not influent their biocompatibility, which allows researchers to design the structures according to their needs, greatly expanding their applications in the life sciences [73].

To verify the safety of DNA nanomaterials in vivo, researchers investigated the distribution and metabolic behavior of DNA nanostructures of different shapes in tumor mice [64]. The results showed that DNA nanomaterials had no significant effect on tumor cell growth, apoptosis, and metabolism-related gene expression. After 12 days of injection of nanomaterial, the body weight of tumor mice did not change significantly compared with that of the control group. At the same time, histopathological analysis showed that these nanomaterials did not cause hepatorenal toxicity. All these results fully demonstrate that DNA nanomaterials have good biocompatibility. In addition, DNA of nanomaterials security in vivo and in vitro was evaluated by other researchers at the same time [74]. Preliminary results show that all kinds of DNA nanostructures have little cytotoxicity. DNA nanomaterials are degraded in vivo and excreted through the metabolic system, which does not lead to the accumulation of toxicity of organs, so it has good biological safety. It is important to note that although the size and shape of DNA nanostructures usually will not affect its biocompatibility, research shows that different DNA nanostructures is greatly different from the absorption and distribution in the body. Therefore, in order to achieve the ideal application effect of biology, we need to prepare a variety of DNA nanostructures and compare their distribution and metabolism in the body, so as to select the most optimal nanostructures for further research.

5.3.2.4 High Chemical Reactivity and Multiple Modification Sites

DNA and functional molecules can be coupled in a variety of ways: covalent modification, nucleic acid hybridization, biotin-affinity interactions, and DNA double-stranded embedding [75]. Based on the precise controllability of DNA nanostructures, it is possible to precisely control the position of functional molecules. The modification sites of DNA tetrahedron are abundant and it is a high capacity carrier. According to the modification position of functional groups or molecules in DNA tetrahedron, there are four main modification methods: vertex, capsule, mosaic, and cantilever [76].

Vertex modification refers to the modification of functional molecules on the vertices of DNA tetrahedron [77]. In addition, bioactive molecules or molecular specific recognition sequence can be modified at the vertices of DNA tetrahedron according to the experimental requirements. In order to synthesize such modified DNA tetrahedron, biological active molecules or specific sequences are often modified at the 5′ or 3′ ends of ss DNA, so that the two ends of four single-stranded DNA are joined at the vertex of the DNA tetrahedron. After that the designed four ss DNA are added to the TM buffer solution in equal quantities, and the DNA tetrahedral framework nucleic acid is formed through one-step annealing hybridization. Studies

have indicated that the aptamer AS1411 has the effect of inhibiting the proliferation of tumor cells [78]. Li Qianshun et al. modified AS1411 aptamer to the vertex part of DNA tetrahedron. The results of this experimental showed that the DNA tetrahedral framework nucleic acid modified with AS1411 at the vertices could inhibit the growth of tumor cells. Moreover, because of the high selectivity of AS1411, it had almost no adverse effect on the growth of normal cells. This research provides a reference for the use of DNA tetrahedron as the carrier of collaborative therapy to deliver a variety of bioactive molecules.

Capsule modification involves wrapping functionalized molecules in a cage at the center of tetrahedral DNA [79]. The cavity in the center of the DNA tetrahedron can be used to encapsulate some nanoscale materials. For example, the DNA tetrahedral framework nucleic acids wrapped in cytochrome C can regulate the entry of apoptotic enzyme activator (Apaf-1). When Apaf-1 and cytochrome C forms a complex, the complex can initiate the cascade reaction of apoptotic protease. Erben et al. constructed a tetrahedron DNA nanostructure whose central cavity structure can contain functional small molecules [80]. They modified cytochrome C to the 5′ end of an oligonucleotide. By changing the sequence of oligonucleotides, the position (internal or external) of cytochrome C relative to the tetrahedron of DNA is regulated. This design can be used to the regulate function of protein. Zhou et al. prepared larger tetrahedral dendritic macromolecules by using AuNPs-wrapped DNA tetrahedron as monomer [81]. By replacing AuNPs with corresponding antigens, this DNA tetrahedral dendritic macromolecule gold nanoparticle conjugation method has promising applications in cancer treatment and immunotherapy.

Mosaic modification refers to the inlaying of functional biological small molecules or groups in the interior of the double helix structure of DNA tetrahedron by means of conjugation [82]. For example, in order to conduct biological imaging analysis or the analysis of the transportation route of DNA tetrahedral nanomaterials, the method of mosaic functionalization is often used to embed fluorescently labeled biomolecules or dyes in the interior of the double helix structure of DNA tetrahedron. Mosaic modification is widely used in the delivery of small-molecule anticancer drugs. The small-molecule anticancer drugs were embedded in the edge of the DNA tetrahedron, and the anticancer drugs were introduced into the target cells in the largest number by virtue of their ability to cross the cell membrane independently, and the cytotoxicity of DNA tetrahedron is little, so as to effectively improve the utilization rate of drugs and at the same time reduce the negative effects on human body to a greater extent. The amount of free doxorubicin (Dox) entering target cells is relatively small, and it has little cytotoxicity to drug-resistant cells. Dox, combined with DNA tetrahedral framework nucleic acids, can enter target cells in a relatively large number and has great toxicity to drug-resistant cells. Using DNA tetrahedron as carrier to transport Dox into breast cancer cells, which is drug-resistant, can better overcome the problem of drug resistance. In order to embed Dox into the double helix structure of DNA tetrahedron, Kim et al. incubated Dox with tFNAs and filtered unloaded Dox with G25 gel [83]. The experimental results show that DNA tetrahedron, as a drug transport system, can significantly inhibit the proliferation of drug-resistant cells and promote cell apoptosis. In summary, tFNAs,

used as a drug carrier to reverse drug resistance of cancer cells in clinic, has a good application prospect.

Cantilever modification means the suspension of functional molecules on the side arms of the DNA tetrahedron. For example, by designing four ss DNA base sequences, the intersection of the 5′ and 3′ ends of the ss DNA is located on the edge of the tetrahedral nanostructure (middle or other non-vertex), where the 5′ or 3′ ends without complementary pairing extend outward for modification of functional molecules or groups [84]. Lee et al. used DNA tetrahedron as the carrier to deliver siRNAs into cells for silencing the expression of tumor-related target genes [85]. At first, they designed six single strands of ssDNA with complementary overhangs at the 3′ ends and then self-assembled the DNA tetrahedron. Among them, each edge of the tetrahedron is suspended in the middle of the uncomplementary paired sequence, which is used to connect the siRNA sequence. Finally, siRNA is immobilized on a cantilever of a DNA tetrahedron into the cell to silence targeted genes.

5.3.3 The Application of tFNAs as a Drug Carrier in Biomedicine Fields

As the carrier of drugs (small-molecule drugs, proteins, nucleic acids, etc.), nanomaterials have been the focus of nanobiology research for accurate drug delivery and controlled drug release. As an efficient and customizable carrier, DNA nano-self-assembly structure has the following advantages: good biocompatibility, targeting, controllability of structure, morphology, and surface chemical modification [86]. Anthracyclines commonly refer to small-molecule anticancer drugs that can be inserted into double strands of DNA to block the synthesis of biological macromolecules in living organisms. Nucleic acid drug molecules can be directly connected to DNA self-assembly structures by base complementary pairing. The study shows that DNA tetrahedron as a drug carrier can make nucleic acid drug molecules play a good role in the body [87, 88]. Other studies have reported that some nucleic acid drug molecules, such as nucleic acid aptamers and siRNA, can enter the cell with the help of DNA tetrahedron and play a role on cancer cells [89–91]. Functional DNA tetrahedron is widely used in biosensors, drug delivery, and biological imaging, due to its advantages of both DNA tetrahedron and specific functional molecules. Research has fully confirmed that DNA tetrahedron as a carrier can realize accurate drug delivery and controlled release in vivo, which has great application potential in the field of nano-diagnosis and treatment.

5.3.3.1 Transport Small-Molecule Drugs for Cancer Therapy

Cancer is a serious disease that seriously affects the health and life of mankind. Timely and effective treatment after the diagnosis of cancer is very important to

improve the survival rate of patients. So far, chemotherapy is still the main treatment for malignant tumors. However, it has shortcomings such as poor solubility, poor cell penetration, poor specificity, large toxic and side effects, and easy to produce multidrug resistance. The cytotoxic effect of traditional chemotherapeutic drugs often causes systemic toxic reaction throughout the body, which reduces the patient's tolerance and leads to the decrease of therapeutic effect [92]. Therefore, it is urgently needed to construct efficient new drug carriers to achieve efficient targeted drug transport, improve drug efficacy, reduce drug toxicity and side effects, and reverse the drug resistance of tumors. Ever since it was proposed, DNA nanotechnology has attracted wide attention in the field of biological detection and drug transportation, especially in the detection, imaging, and treatment of tumors, because of its structural programmability, good biocompatibility, no obvious cytotoxicity, and immunostimulation [93]. Doxorubicin (Dox) is a first-line antitumor drug, which can inhibit many kinds of tumors. Dox works by embedding bases into DNA, preventing mRNA formation and thus inhibiting DNA and RNA synthesis. Doxorubicin's mechanism of action is to prevent the formation of mRNA by embedding DNA double strand, thus inhibiting the synthesis of DNA and RNA. It has the strongest inhibitory effect on RNA [94]. It is a cyclically nonspecific drug which has a killing effect on tumor cells in different growth cycles. DNA nanostructure is used as a carrier to carry Dox for antitumor, which is of great significance for improving the efficacy of Dox, reducing side effects and overcoming cell resistance. Using DNA tetrahedron as Dox carrier, Yan et al. obtained Dox-tFNA complex by incubating the synthesized DNA tetrahedron with Dox, and the Dox load efficiency was greatly improved [95]. The results indicate that the Dox-tFNA complex is not only cytotoxicity to human breast cancer cells but also cytotoxicity to Dox-resistant cancer cells. Furthermore, in comparison with free Dox, the killing effect of Dox-tFNAs complex on cancer cells was significantly improved and the clearance efficiency was decreased.

Targeted therapy means that drugs can be combined with targeted tumor cells to inhibit tumor growth. It is one of the important technical paths to overcome the toxicity of traditional chemotherapy system. MUC1, a kind of transmembrane glycoprotein, is overexpressed in a variety of cancer cells. MUC1 also plays a certain role in tumor growth, metabolism and metastasis [96]. In addition, the structure of MUC1 expressed in tumor cells is different from that expressed in normal tissue cells, so MUC1 has become a target molecule with certain tumor characteristics, which provides a biological basis for its application in targeted therapy. Studies have shown that monoclonal antibody against MUC1, vaccine, small-molecule ligand, and other methods can achieve certain initial effects in tumor inhibition experiments in vitro and in vivo [97]. It has been shown that tFNAs can be used as a drug deliverer to load the antitumor drug Dox.

Paclitaxel (PTX) is a diterpenoid alkaloid compound separated and purified from the bark of *Taxus cuspidata*. It is the most outstanding anticancer drug found in nature, which has been widely used in the therapy of ovarian cancer, breast cancer, lung cancer, and partial head and neck cancer. Due to the rapid proliferation of tumor cells, it can play an antitumor role by inhibiting the mitosis process of tumor cells

[98]. PTX, as a new anti-microtubule drug, promotes the polymerization of microtubules, thus preventing the depolymerization of microtubules and disrupting the normal function of cells, thus inhibiting the mitosis of tumor cells and promoting cell apoptosis. Although the clinical treatment is very good, the wide application of PTX is limited by multidrug resistance. Recently, tFNAs have been thought as promising drug nanocarriers. In order to overcome paclitaxel resistance, tFNAs are used to deliver PTX into MDR cells as an efficient vehicle. Xie Xueping et al. incubated synthesized tFNAs and PTX to construct a PTX/tFNA drug transport system [99]. They compared the toxic effects of PTX/tFNA drug delivery system with that of free PTX on lung cancer cells and explored the mechanism by which PTX/tFNA drug delivery system reverse drug resistance. This study shows that PTX/tFNA drug delivery system can inhibit the growth of multidrug-resistant cancer cells significantly because of highly efficient load rate of PTX by the drug system. In addition, this study shows that PTX/tFNA drug delivery system has great potential in revers drug resistance and tFNAs have great application prospects in drug delivery and the treatment of multidrug-resistant cancers.

5.3.3.2 Transport Functional Nucleic Acids

Functional nucleic acid refers to nucleic acid molecules with special functions, including two categories: one is the DNA/RNA with similar properties to antibodies, which can specifically bind to the target molecule, called nucleic acid aptamer; another is nucleic acid molecule with similar catalytic activity to proteases is called DNAzymes [100]. Functional nucleic acid has the advantages of strong binding force, good selectivity, wide range of targets, good biocompatibility, easy synthesis, and easy functional modification. Functional nucleic acids include aptamer, antisense oligonucleotides, small interfering RNA (siRNA), microRNA, and other nucleotide sequences with special functions, which are widely used in the diagnosis and treatment of diseases. However, the unmodified functional nucleic acid molecules are easy to be degraded by various nucleases in the physiological environment, and the carrier needs to be able to protect the integrity of nucleic acid molecules for a long time and extend the circulation time of nucleic acid drugs in the body, so as to ensure that the nucleic acid drug molecules reach the target cells and play a role [101]. DNA tetrahedron and nucleic acid drug molecule homology is a good nucleic acid drug carrier. Since both the carrier and the drug are nucleic acids, it is convenient to carry the drug molecules by nucleic acid hybridization or embedding [102, 103].

Antisense Oligodeoxynucleotide

Antisense oligonucleotides (AONs) are nucleic acid molecules that inhibit the expression of a target gene by specifically binding to its DNA or mRNA in a sequence. By binding with specific target sequences of mRNA or DNA, antisense

oligonucleotides can block mRNA transcription and translation, regulate the information transfer from gene to protein, and inhibit protein expression. The data show that oligonucleotides designed with 15–20 base sequences can specifically target any specific single gene in the human genome [104]. Since 1978, when Zamecnik and Stephenson et al. first reported that antisense oligonucleotides could inhibit the replication of chicken sarcoma virus, a large number of studies have confirmed the ability of antisense oligonucleotides to specifically inhibit gene expression, and their great potential in disease treatment has been gradually recognized [105]. Antisense oligonucleotides targeting specific oncogenes, growth factors and their receptors, and signaling pathways have been widely used as a new tool for in vivo and in vitro studies and as a promising drug for the therapy of diseases such as diabetes, asthma, cancer, and viral infections, attracting great interest [106]. However, antisense oligonucleotides are rapidly degraded when they enter the cell without modification, and the degradation rate is greatly reduced after modification or carrier binding. At present, most carriers are cationic particles and liposomes, which are cytotoxic. tFNA is considered to be the most ideal nano-drug carrier because of its editable, easy modification, and biocompatibility. Zhang Xiaolin et al. designed antisense oligonucleotides targeting the c-met gene and constructed antisense oligonucleotide-DNA tetrahedral drug delivery system for the treatment of cancer. The results showed that DNA tetrahedron could successfully transport antisense oligonucleotides into cells and antisense oligonucleotides could bind to the target gene mRNA, inhibit the expression of related proteins, and finally achieve the purpose of inhibiting tumor cell proliferation and promoting cell apoptosis [107]. In addition, Keum et al. constructed DNA tetrahedrons with antisense characteristics by using five oligonucleotides, which can bind to targeted mRNA and block the expression of some specific genes after entering cells [90]. The results indicated that, in comparison with linear DNA, DNA tetrahedrons show higher ability of cell uptake and gene silencing.

Antisense Peptide Nucleic Acid

Antisense peptide nucleic acid (asPNA) is a kind of synthetic DNA analogue, which has higher water solubility, stability, and base specificity compared with nucleic acid. The antisense peptide nucleic acid and double strand of DNA can form a triplet structure, which can block gene transcription and translation and inhibit gene replication by inhibiting the extension of DNA primers [108]. Antisense peptide nucleic acid can downregulate or inhibit expression of the target genes in gene replication, transcription, and translation, so as to achieve the goal of disease treatment. Antisense peptide nucleic acid, as third-generation gene therapy agent, has been widely used in vitro to inhibit the proliferation of tumor cells and the treatment of bacterial or viral infections. However, without the help of any drug delivery system, asPNA has a hard time crossing cell membranes [109, 110]. The further improvement of the intake of asPNA and the ability of asPNA to enter the nucleus has been a key issue affecting its wide clinical application. The traditional

drug delivery system has many disadvantages, such as protease sensitivity, insufficient bioavailability, poor effective targeting, lack of cell specificity, immunogenicity to animal cells, and potential cytotoxicity. It is difficult for the unmodified synthetic antisense peptide nucleic acid to pass through the bacterial cell wall and be absorbed by the bacteria [111]. Therefore, bacterial cell wall is the biggest obstacles to the development of antisense peptide nucleic acid as a therapeutic antimicrobial agent. The cell penetrating peptide covalently connects with the antisense nucleic acid to facilitate the asPNA to penetrate the bacterial cell wall and transfer it into the cell. Although they are very effective carriers of asPNA, they can't specifically identify bacterial cell walls and can show growth inhibition at high doses. In Zhang Yuxin's study, a complex asPNA-tFNAs drug delivery system was constructed. In this study, with tFNAs as the carrier, asPNA with high specificity, high affinity, and specific targeting of ftsZ gene was transported into bacteria, in order to inhibit the expression of this gene and achieve the purpose of bacteria inhibition [112]. Guided by the principle of base complementary pairing, asPNA replaces part of the tFNAs sequence without changing the original structure, shape, size, or excellent carrier properties of the DNA tetrahedron. The results showed that the asPNA-tFNA vector system could penetrate the bacterial cell wall and carry antisense peptide nucleic acid targeting specific genes to block the expression of the specific genes, thus effectively inhibiting the growth and proliferation of MRSA bacteria. John has designed a self-assembling DNA tetrahedral framework nucleic acid as the drug carrier and incorporated a targeted asPNA in its structure to penetrating the cell wall of bacteria. This research show that the minimum inhibitory concentration (MIC) was reduced when the asPNA is carried by the DNA tetrahedron, contrasting with no reduction in MIC when PNA4 is used alone [109]. The drug delivery system has the ability to penetrate the bacterial cell wall and deliver the targeted synthesis of asPNA, which has a wide range of optimization selection and potential application.

Aptamer

Aptamer is a small fragment oligonucleotide sequence or short polypeptide selected in vitro, which can bind with the corresponding ligand and has the advantages of high affinity and strong specificity. The appearance of aptamers provides a new research platform for chemical biology and biomedical science to identify nucleic acid aptamers quickly and efficiently [113]. In 1990, Tuerk and Ellingtong were the first to screen the specific RNA aptamers of phage T4DNA polymerase and organic dye, respectively [114, 115]. Since the concept of the aptamer was put forward in the 1990s, researchers have been devoting themselves to the study of the aptamer and found that the aptamer has many advantages. First of all, adapters have the advantages of short detection cycle, low detection limit, high affinity and strong specificity, which make adapters have larger surface area and a large number of receptor binding sites.The aptamer can combine with the target material based on van der Waals forces, hydrogen bonds and hydrophobic effects to form three dimensional

structures such as helix, hairpin, stem ring, convex ring, clover and pseudo structure. Secondly, compared with antibodies, the selected aptamers are easy to be synthesized in vitro in large quantities, with good repeatability and high stability, and are easy to be stored [116]. Since then, aptamers have been widely used in cell imaging, drug development, disease treatment, and microbial detection. Researchers have developed a variety of anticancer drugs, and nanomaterials are widely used for drug delivery, but they have the disadvantages of poor biocompatibility, unable to deliver in a targeted manner and harmful to other cells. The aptamer can specifically bind to the target, and the nanomaterial-specific aptamer complex can realize the targeted drug delivery in the cell. Liu et al. constructed an icosahedral nanomaterial, which, after binding with the specific aptamer DNA, can carry doxorubicin and deliver it to the lesion location in a directional manner, specifically leading to the death of epithelial cancer cells [117].

AS1411 is a guanine-rich aptamer, which can form G-4 chain structure, can specifically bind to nuclides, and has many special biological activities. Nucleolinins are highly expressed on the nucleus and the surface of the tumor cell membrane, and AS1411 can enter the nucleus by the shuttle action of nucleolinins in the cell. At the same time, AS1411 can inhibit DNA replication; make the cells stay in the S phase, thus inhibiting cell proliferation; and promote cell apoptosis by interfering with the binding of nuclides and bcl-2 [118]. AS1411 has a great prospect in the treatment and diagnosis of cancer. Because of its good biocompatibility, good stability and modifiability, and simple synthesis method, DNA tetrahedron has a great prospect in drug loading, tumor therapy, and other fields. DNA tetrahedron can be used as nanometer drug carrier material with good biocompatibility [119]. Li Qianshun et al. modified AS1411 aptamer at the vertex of DNA tetrahedron to study the specific recognition effect of the drug delivery system targeting tumor cells [120]. The results showed that Apt-tFNA complex could enter McF-7 cells in large quantities and accumulate in the nucleus. However, relatively few Apt-tFNAs can enter into normal cells L929, suggesting that AS1411-modified tFNAs are an effective drug delivery vector targeting tumors. However, the amount of Apt-tFNA complex entering normal cell L929 was relatively small. These results suggest that tFNAs modified with AS1411 is an effective drug delivery vector targeting tumors cells. Bermudez et al. studied the effect of DNA tetrahedron modified with AS1411 aptamer on HeLa cells. The results showed that AS1411-tFNA drug delivery system was more easily absorbed than the AS1411 aptamer alone and it could inhibit the proliferation of HeLa cells significantly for up to 24 h [121]. However, AS1411-tFNA complex had little effect on the growth of noncancerous cervical cells. This result demonstrated that the AS1411 aptamer suspended on the tetrahedron of DNA is more easily recognized by the receptor and inhibits the growth of cancer cells through specific uptake and absorption.

siRNA

RNA interference (RNAi) refers to the phenomenon of gene silencing caused by degradation of target mRNA or inhibition of translation. The advantages of specificity, efficiency, and stability of RNA interference technology make it a hot topic in mechanism research in biomedical field in recent years, and it is widely used in cancer mechanism research and cancer therapeutic drug research [122, 123]. Single-stranded or double-stranded RNA containing 21–23 bases, namely, small stranded interfering RNAs (siRNAs), can effectively and specifically block the expression of homologous genes in vivo through RNA interference, promote homologous mRNA degradation, and induce cells to show the phenotype of specific gene deletion. siRNAs combine with proteins, such as eLF2c, Gemin3, Gemin4, and so on, forming the RNA-induced silencing complex (RISC). RISC can specifically recognize and degrade target mRNAs under the antisense chain of siRNAs [124–126]. Binding of siRNAs to target mRNA sites is a highly sequentially specific process, following the principle of complementary pairing of the Watson-Crick bases. Wilkins et al. found the regulatory program of RNA interference on *Caenorhabditis elegans* genes, and they found that RNA interference is the innate antiviral immune defense mechanism of *Caenorhabditis elegans*, and it is a conserved sequence-specific post-transcription gene silencing mechanism in the evolutionary process [127]. The discovery of RNA interference opens the door to the application of siRNAs therapy. siRNAs have shown great advantages and potential in basic research in molecular biology and in the treatment of viral infections, tumors, and genetic diseases. Tumors are often malignant growths of cells caused by overexpression of proto-oncogenes or loss of control of tumor suppressor genes. siRNAs can be used to specifically block the expression of these genes in vivo for therapeutic purposes. The development of RNA interference technology is of great significance for elucidating signal transduction pathways and discovering new drug targets, but their safe and effective introduction methods and methods of stable expression in vivo, as well as the expression of target gene downregulation at mRNA and protein levels caused by RNA interference effect need to be further studied [128]. The short time in vivo and short half-life in plasma of siRNA make its application limited. The lack of safe and effective vectors restricts the application of siRNA. Anderson et al. introduced siRNA into live nude mouse tumor model by using DNA tetrahedral nanomaterials as the carrier to inhibit the expression of target genes for tumor therapy [85]. The team suspended the siRNA on an arm of the DNA tetrahedron by means of base complementary pairing, which transported the DNA tetrahedron modified with the siRNA to the lesion site through the ligand connection with the cancer cell receptor. The results show that DNA nanostructure can improve the stability of RNA molecules, thus significantly improving the efficiency of RNA interference. Besides, Kim et al. used DNA tetrahedron as the transporter of siRNA to silence some genes in cancer cells, which modified folic acid molecule on the tetrahedron to promote the process of RNA interference [129]. In addition, blood circulation time of DNA tetrahedron modified by siRNA is longer than that of free siRNA. Since gene silencing can be carried out by transferring siRNA through

tFNAs, this drug delivery system can not only silence tumor target genes by transferring siRNA but also be used to treat other gene-related diseases.

Num, Dpt.

CpG oligonucleotide sequences are widely found in bacterial genomes and a small amount in mammalian genomes, which are active agents to induce innate immunity and acquired immune response. It is regarded as the signal of pathogen invading the immune system and is recognized by toll-like receptor 9 to induce the immune response [130]. CpG oligonucleotide sequences can be used as a potential therapeutic DNA and immune adjuvant in the adjuvant treatment of infectious diseases, cancer, allergies, and asthma. However, unmodified CpG oligonucleotide sequences is generally easy to be degraded by nuclease, with low cell uptake rate, requiring high dose and repeated administration, which severely limits the application of CpG [131]. Since natural CpG sequences are easily degraded by nucleases, the development of nontoxic nanocarriers with high transport efficiency is of great significance to improve the stability and clinical treatment effect of CpG. In recent years, the development of DNA nanotechnology to solve the problem of nucleic acid drug delivery provides a new tool. A large number of research reports have shown that nanomaterial-loaded CpG nucleic acid drug has high activity, low toxicity and good biocompatibility, and is expected to become a new immunotherapy drug for the prevention and treatment of related diseases [132–134]. Fan Chunhai's research group first used DNA tetrahedral nanostructures as CpG carriers. The DNA tetrahedral nanostructures in this experiment were assembled using four single-stranded DNA strands through a simple annealing reaction [135]. These four vertices of the tetrahedron extend 1–4 CpG oligonucleotide sequences. The cell uptake of CpG-tetrahedron was detected by fluorescence imaging and flow cytometry. The results showed that single-stranded CpG DNA was difficult to be absorbed by cells, but CpG-tetrahedron could be widely consumed by cells, proving that the tetrahedron structure plays an important role in promoting cell uptake. In addition, a series of experiments have demonstrated that the tetrahedral nanostructure can enhance the stability of CpG DNA inside and outside the cell. Moreover, they used CpG-modified tFNAs to study the immune activation of macrophages (RAW 264.7). Inflammatory cytokines stimulated by CpG-modified tFNAs, such as TNF-α, IL-6, and IL-12, were much more numerous than single-stranded CpG sequences. This study indicated that tFNAs can increase CpG load, thus inducing immune response more obviously, suggesting that tFNAs can be used as a good carrier for immunotherapy.

5.3.3.3 Transport Peptides and Proteins

Peptides and proteins, including vaccines, immunoglobulin, and enzymes, can be used as drugs for the prevention, treatment, and diagnosis of diseases. Peptides and

proteins are endogenous substances of human body or developed for biological regulators in vivo [136]. Peptides and proteins act by promoting or inhibiting physiological and biochemical processes in the human body or in bacterial viruses. Advantages of peptides and proteins include low side effects, high potency, and strong pertinence, and they will not accumulate in the body and cause poison [137]. Polypeptides and protein-based drugs are the most active and the fastest developing drugs in the field of pharmaceutical research and development, and they are among the most promising industries of the twenty-first century. However, there are also many problems in the application of peptides and proteins drugs. For example, the structure of protein drugs is unstable, and it may be inactivated due to a variety of complex chemical degradation and physical changes in the in vivo and in vitro environment [138]. In addition, protein drugs have many disadvantages, such as short half-life, high clearance rate, large molecular weight, poor transmittance, vulnerability to the destruction of enzymes, bacteria and body fluids, and low bioavailability of non-injectable drugs. Studies have shown that peptides and proteins can bind to DNA nanostructures and enter cells [136, 139, 140]. Yan's team reported on the construction of nanoscale vaccines using DNA tetrahedrons. By the specific binding of biotin-avidin, the biotin-modified DNA tetrahedron loaded a model antigen of streptomycin into mice [141]. The tetrahedral antigen complex can induce a strong antibody response in mice continuously and steadily, while the tetrahedral or antigen alone does not stimulate any immune response. Catabolite activator protein (CAP) is an important transcription factor that regulates over 100 genes. Recently, Turberfield's team provided a template for DNA-protein assembly by designing CAP recognition sites on the side chains of DNA tetrahedrons and assembling caps into tetrahedrons in a non-covalent manner [142]. Zhao et al. found a fast and efficient method for enzyme inclusion: first, attach a single enzyme molecule to half of the DNA cage, and then combine the two half-cage structures to successfully load the enzyme into the DNA cage cavity [143]. They also recorded glucose oxidase (GOX) and HRP in a DNA cage at a ratio of 1:1. As a carrier of proteins, DNA nanocages (such as tFNAs) can protect proteins from the influences of external environment (such as the biodegradation of proteins by enzymes), to protect the activity of proteins and improve the stability of proteins.

5.3.3.4 Transport Multiple Drug Molecules

When two or more drugs are used together, if they act in the same direction, the effect of mutual enhancement is called synergy. Based on the synergistic effect, the therapeutic effect can be enhanced when the DNA tetrahedron co-transports multiple drug molecules which act in the same direction [144]. In many cases, the actual treatment of a disease requires the synergistic action of multiple drugs for composite therapy, which requires the nanomaterial delivery system to simultaneously deliver multiple drug molecules and maintain their respective activity. Because DNA tetrahedral modification sites are abundant, it can carry different drug molecules at the same time. To enhance the effect of killing tumor cells and drug targeting, Dai

Phuendong et al. conjugated MUC1 nucleic acid aptamer (Apt) with DNA tetrahedron to construct a targeted doxorubicin delivery system [145]. This study also evaluated whether the vector system could bind to MUC1-positive tumor cells specifically and be cytotoxic to MUC1-positive tumor cells. MUC1 aptamer and DNA tetrahedron can be used to construct the drug carrier with targeted action. Since MUC1 aptamer can specifically identify tumor cells, it improves the targeting effect of DOX and the killing effect on tumor cells. The results showed that Apt-TDN-Dox drug carrier system could produce significant cytotoxicity to MUC1-positive cancer cells, which is more effective than the free Dox. In addition, Liu et al. used DNA tetrahedron to co-transport CpG and streptavidin. CpG sequence can be used as an adjuvant to enhance the efficacy of vaccine due to its strong immune stimulation activity, so the antigen-CpG-DNA tetrahedron complex can continuously induce a stronger immune response [146]. Therefore, the coordinated delivery strategy is of great significance in reducing the dosage of drugs and improving the therapeutic effect, which is worthy of further exploration by researchers.

5.3.4 The Current Problems of tFNAs as a Drug Carrier in Biomedicine Fields We Faced

The simple chemical synthesis connects functional molecules (inorganic small molecules, biological macromolecules, inorganic nanoparticles, etc.) to the DNA tetrahedron to form multifunctional DNA composites. These composites have shown great potential in the field of nano-diagnosis and treatment. However, there are still some problems in the application of DNA tetrahedron to living system [147–149]. For example, high cationic concentrations are required for DNA self-assembly structures to maintain structural stability; DNA's ability to self-assemble structures across cell membranes is limited; After DNA self-assembly structure enters biological system, it is easily degraded by various biological enzymes in serum. These problems restrict its application to some extent. Therefore, it is very important to improve the stability of DNA self-assembly structure and improve kinetic performance and cellular uptake efficiency of DNA self-assembly structure.

5.3.4.1 Improves the Stability of DNA Tetrahedron In Vivo

As mentioned above, we believe that DNA tetrahedron has great application prospects in the biomedical field, including the use of DNA tetrahedron alone to regulate the biological behavior of cells, and the use of DNA tetrahedron as a carrier to achieve targeted therapy, which requires DNA tetrahedron to have certain stability in the systemic circulation and to avoid immune recognition to some extent [150]. The application of DNA tetrahedron to living system requires special consideration of its stability at low magnesium ion concentration, 37 °C, and nuclease conditions. Fetal

bovine serum is a mixture used in cell and tissue culture usually. It contains a variety of biological enzymes that have a strong degradation effect on nucleic acid molecules. However, Bermudez's team confirmed that the degradation rate of DNA tetrahedral structure in FBS was only 1/3 of that of a single strand [151]. They believed that this was mainly because the folded DNA structure affected the binding of enzymes to DNA strands, thus preventing the degradation of DNA strands by biological enzymes. In a solution containing 80% mouse serum, tFNAs were completely degraded after 12 h. However, in in vivo circulation, there are more adverse factors, including enzyme degradation, protein absorption and conditioning, RES clearance, etc., which put forward higher requirements for the stability of tFNA's in vivo circulation. We need to use various methods to hide the surface features of DNA nanostructures in order to inhibit nonspecific clearance in vivo. The researchers added a variety of polymers, cationic particles, and so on to the DNA structure to coat the nucleic acid, thus achieving better stability and drug carrying capacity. Among the methods, polyetherimide (PEI) is a classic method. With proper PEI and nucleic acid ratio, the preparation can be completed after incubation at room temperature [152]. The synthesized complex has high transfection efficiency in the gene transfer field and can promote the lysosome escape of nucleic acid through proton sponge effect. However, due to the cationic characteristics of PEI, the biocompatibility is poor, in recent years, the academic community has also proposed a variety of PEI modification or replacement materials, so as to improve the cytotoxicity of PEI.

In addition, DNA chain modification can also inhibit the degradation of DNA by nucleases. Sleiman's team modified a C12-alkyl chain on the DNA strand, using the complex to connect the DNA cubes to human serum albumin (HSA) to form a nanoscale superstructure that remained stable in the serum for 22 h, while DNA cubes without HSA remained stable in the serum for only a few minutes [153]. Dietz et al. studied the influence of temperature on the stability of DNA self-assembly structure, and they found that the self-assembly structure of DNA constructed by multilayer origami has a strong stability, which can be stable at more than 50 °C, and multilayer origami can effectively inhibit the degradation of nucleases [154]. These results suggest that DNA dense accumulation, chemical modification of DNA strands, and end protection are conducive to the good stability of DNA nanomaterials in the medium containing enzymes and at high temperature. In addition, the use of virus liposome garment at the end of the shell, such as package structure of DNA self-assembly, also can improve the DNA stability of nanomaterials in the physiological environment. Due to the complex internal environment of the organism, it has not been determined whether DNA nanostructures can maintain stability for a long time under the condition of low ion concentration in vivo. At present, the research of intelligent drug delivery is limited to in vitro, not involving cell and in vivo applications. How to develop and construct DNA nanomaterials that can cope with the complex biological environment, effectively complete the loading, transportation and unloading of macromolecules, and truly realize the biological motors in nature, so as to truly apply DNA nanomaterials to clinical medicine, still needs further exploration and solution by researchers.

5.3.4.2 Improves the Cell Uptake Efficiency of DNA Tetrahedron

Turberfield team first explored cells can ingest DNA self-assembly structure in without the help of transfection reagents [155]. They modified Cy3 and Cy5 fluorescence on the two edges of the DNA tetrahedron, respectively, and the result confirmed that the DNA tetrahedron could enter the cell without transfection reagent through fluorescence imaging. The fluorescence resonance energy transfer between Cy3 and Cy5 showed that the tetrahedron entering the cell still maintained a good integrity. Later, Mirkin's team constructed "spherical nucleic acid" nanostructures by densifying DNA strands on nanoparticles, which could also enter cells efficiently without transfection agents, suggesting that three-dimensional DNA nanostructures were more conducive to cell uptake than single strands [156]. Studies have shown that the DNA tetrahedron enters the cell through receptor-mediated endocytosis and then enters the lysosome of the cell, which can remain stable in the cell for at least 8 h. Use the function of target molecules can enhance cell active uptake of DNA nanostructures. Mao et al. increased the uptake of DNA nanostructures by modifying folic acid on DNA nanostructures to make folate receptors highly expressed on the surface of tumor cells [157]. Nucleic acid aptamers can be directly connected to DNA nanomaterials through DNA synthesis, which can not only improve the uptake efficiency of DNA nanomaterials but also have specificity. "Hidden" DNA nanostructures are also an effective way to promote cell uptake of DNA nanomaterials. Perrault and Shih used viral capsid proteins and liposomes to wrap the DNA octahedron, which reduced its immunogenicity by 2 times and increased its biological effect by 17 times [158, 159]. In summary, compared with linear DNA chains, the symmetry, size, and functional modification of three-dimensional DNA nanostructures have obvious effects on the uptake efficiency of cells. Moreover, there is no definitive answer to the question of how DNA nanostructures interact with cells. The study of cellular uptake of DNA nanostructures is crucial for the subsequent biomedical applications of DNA nanostructures.

5.3.4.3 Improve the Synthetic Yield and Reduce the Synthesis Cost of DNA Tetrahedron

At present, using DNA nanostructure as drug carrier also has some disadvantages, such as low yield and high cost. First, although the construction of a rich and diverse nanostructure has been achieved, the mechanism of DNA assembly is still not very clear, and the thermodynamics and dynamics of the assembly process are still unclear [160]. Because of the unclear assembly mechanism, the mismatch problem in the assembly process is difficult to solve, and it is also difficult to get a stable high yield. Therefore, it is still a difficult problem to explore the assembly mechanism and obtain high yield and stable assembly. Secondly, DNA nanomaterials need to be purified. To solve this problem, the purification methods such as gel electrophoresis, high-performance liquid chromatography, and density gradient centrifugation were

developed to improve the purity of the product to a certain extent [161]. In the end, although DNA nanotechnology has been applied to biomedicine, electronic science, and other fields, the high preparation cost of DNA nanostructure restricts the practical application of DNA nanotechnology [162]. Compared with other nanomaterials that can be prepared on a large scale, the synthesis cost of nanomaterials constructed from DNA molecules is still high. Therefore, the development of cheaper and more convenient means of DNA amplification is another challenge to be tackled. In recent years, with the development of DNA synthesis technology, the cost and price of commercial synthesis are rapidly decreasing [159, 163]. It is believed that the cost of synthesizing DNA nanostructures on a large scale commercially will fall to a level that medical applications can afford in the near future.

5.4 Conclusion

This paper reviews the concept of DNA nanomaterials and the development of DNA nanotechnology. In this paper, the synthesis and physicochemical characteristics of DNA tetrahedron, which is the most widely used and most studied of DNA nanomaterials, as well as the research progress of DNA tetrahedron for intelligent drug delivery, are also described in detail. By loading small molecules and protein drugs into DNA tetrahedron, the drug resistance of some drugs was reversed, and the problems of easy degradation, poor water solubility, and short retention time of some drug molecules were solved. Translocation of functional nucleic acid molecules by DNA tetrahedron is a new concept of cross-domain generation. Based on the compatibility and homology between drug molecules and carriers, DNA tetrahedron provides an incomparable platform for the transport of nucleic acid drugs. The intelligent drug delivery system based on DNA tetrahedron realizes the controlled release of drug molecules at the targeted sites, significantly improves the efficacy, and reduces the side effects. Functional DNA tetrahedron nanomaterials have the advantages of simple self-assembly, high yield, stable structure, good biocompatibility, and not easy degradation. They show a wide range of applications and good prospects in biosensors, separation analysis, biological imaging, and drug delivery.

At present, the application of DNA tetrahedral framework nucleic acids as drug carriers still faces many challenges. First, in the field of diagnosis and treatment of disease, at present, mice are still the main subjects of in vivo experiments on DNA tetrahedral framework nucleic acids, which still face many challenges nanostructures as drug transporters is one of the main targets of future research. Secondly, the exact mechanism by which cells take up the tetrahedron of DNA is still unclear. Recent studies have shown that the size, shape, charge, and cell type of DNA tetrahedral framework nucleic acids affect their cellular uptake, intracellular transport, and eventual destination. At present, little is known about the final transformation of DNA tetrahedron in cells, which is an important question for future research. In addition, the main challenges are to further improve the yield and the stability of

DNA tetrahedral framework nucleic acids and explore the cellular uptake mechanism.

Despite many problems, DNA nanotechnology is still an invention with great potential. We have reason to believe that the development of DNA nanotechnology will bring new light to human beings in DNA chips, nano-devices, biomedicine, and other aspects and will certainly promote the progress of related fields. With the development of related researches, DNA nanostructures will have a broader development prospect in the application of drug transporters and intelligent drug carriers. DNA nanotechnology is still evolving as a cross-cutting science, drawing strength from fields such as physical chemistry, optics, and electronics. We have reason to believe that eventually it will show up in the biomedical field and be widely used.

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Chapter 6
Research Progress on Antibacterial Application with Nucleic Acid and Nucleic Acid Materials

Yue Sun and Yunfeng Lin

Abstract Typically, the abuse of antibiotics leads to the emergence of multidrug-resistant pathogens. Abusing antibiotics reduces the killing effect of the antibiotics, which directly impacts the efficacy; as a result, the difficulty of treatment also increases. Developing alternative treatment strategies is urgently needed to reduce the mortality and incidence rate of drug-resistant bacterial infections. Over the past two decades, studies have shown that nanomedicine has the potential to be applied as an antibacterial agent. It has become a novel tool against resistant bacteria. Reportedly, metal and metal oxide nanoparticles (NPS) are the most common nanoparticles. Until recently, numerous scholars used DNA nanostructures alone or functionalized with specific DNA sequences to achieve antibacterial purposes to treat severe bacterial infections. It is a potential therapeutic method with significant potential to target and eliminate antibiotic-resistant bacteria. This paper reviews the dimensions, the underlining mechanism of multidrug bacterial resistance, and the current research progress of nanomaterials based on nucleic acid in the application of antibacterial treatment.

Keywords DNA nanostructure · Antibiotic · Bacterial · Nucleic acid aptamer · Antisense oligonucleotide (ASOs) · Antibacterial peptide (AMP) metal nanoparticles

6.1 Introduction

The abuse of traditional antibiotics has led to the emergence of multidrug resistance (MDR) strains, and its severity cannot be ignored [1, 2]. Antibiotic resistance has become a significant threat to the health of the global public, and it could lead to a situation where people die because of simple infections. Finding new and

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unconventional antibiotics or substances is a crucial challenge. The manufacture of a majority of the antibiotics currently available on the market took place in the twentieth century. Since 2000, very few new antibacterial drugs have been developed, and the number of new drugs has been limited [3, 4]. Some strains exhibit resistance to a variety of conventional antibiotics, which limits the treatment options for drug-resistant bacterial infection. In addition to the high cost, the application of high-dose antibiotics has a lot of side effects on the body [5, 6]. Studies have indicated that the treatment cost of MDR infection varies significantly due to the drug sensitivity of the infected strains. In the United States, the treatment cost of patients with methicillin-resistant *Staphylococcus aureus* (*MRSA*) infection is much higher than that of patients with methicillin-sensitive *Staphylococcus aureus (MSSA)* infection [7, 8]. The cost of treatment for *MSSA* infection is about $16,000; meanwhile, the cost of treatment for *MRSA* infection is about $36,000. In China, the average aggregate medical cost of MDR infected patients is ¥ 131,801, while that of patients without MDR pathogens is ¥ 41,600 [9, 10]. Besides, the length of stay in patients with drug-resistant bacterial infection is prolonged, while the incidence rate and mortality rate increase. For example, *MRSA* infection is more likely to affect the lungs, blood, and urethra, while the *MSSA* infection is more likely to affect the bones or joints, eyes, ears, nose, skin, or soft tissues. Therefore, the mortality rate of *MSSA* infection was 11.5%, while that of *MRSA* infection was 23.6% [7]. Gram-positive and Gram-negative bacteria have different cell wall structures (Fig. 6.1) and different ways of drug resistance. The principal mechanisms of antibiotic resistance include enzyme degradation or enzyme modification of antibiotic molecules, the outflow of antibiotics from bacterial cells through active efflux pump, and transformation of antibiotic targets that prevent antibiotic binding [11]. Presently, the research on antibiotic-resistant bacteria involves the combination of antibiotics and the inhibition of enzyme degradation or modification of antibiotic binding sites to reduce antibiotic resistance.

The nucleic acid is an essential genetic material in pathogenic microorganisms. It plays a vital role in a series of crucial life phenomena, such as growth, heredity, and variation. The application of nucleic acid in bacteria detects the type of pathogenic microorganism and the existence of drug-resistant gene by detecting nucleic acid [12, 13]. Strengthening the endocytosis or adhesion of antibiotics is another option to reduce the outflow of antibiotics from bacteria that are insensitive to antibiotics [14–17]. Recently, the use of nanoparticles and other antibacterial materials to control bacteria has attracted the worldwide attention of researchers. The morphological and physicochemical properties of these materials make them of interest, including high specific surface area and volume ratio, the successful application of physical and chemical properties to other applications [18], dissociation reactions triggered by different environments over time and efficacy, and pathogens. Additionally, the surface charge on these nanoparticles can promote a combination with the opposite surface charge of bacteria, which produces effective antibacterial activity. Similarly, due to the insolubility of these antibacterial nanoparticles and their close interaction with microbial membranes [19], their practical lifespan and durability in antibacterial applications are very promising. The application of nucleic acid materials in the field of antibacterial primarily focuses on target detection, instead of the antibacterial area. However, with the development of nucleic acid materials, an increasing number of

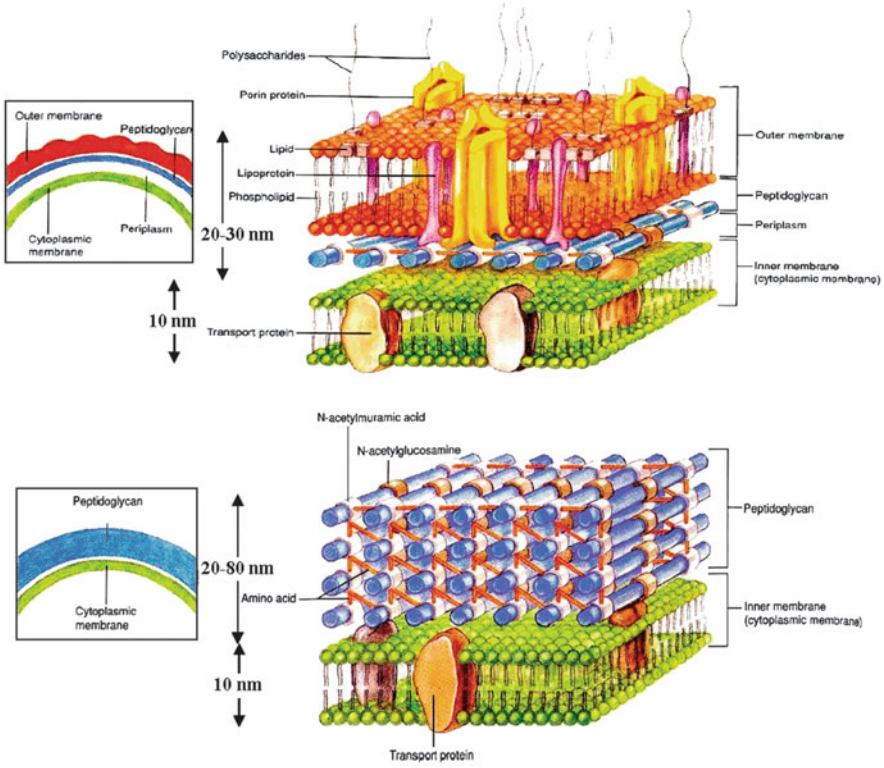

Fig. 6.1 Comparison between gram-positive and gram-negative bacteria cell wall structures. Copyright 2008, Royal Society of Chemistry [20]

researches are directed in the study of antibacterial characteristics. In this chapter, the application of nucleic acid and nanomaterials in the antibacterial field is reviewed. Concurrently, the current situation and the existing obstacles in the application of antibacterial therapy are also discussed.

6.2 Nucleic Acid Aptamer

The DNA spatial structure is usable for drug release. Compared with simple nucleic acids, the DNA spatial structure can easily penetrate into cells and transfer drug molecules without any ligands or transfect agents. Besides, it also has high flexibility of different sizes, allows an increased capacity loading rate, and enhances the drug molecules' killing efficiency. Researchers utilize DNA spatial structures to control and transfer various drug molecules or nucleic acid sequences to living cells, including anticancer molecules, siRNA, antibodies, peptides, photosensitizers, and so on [21]. Gene silencing prevents the expression of a specific gene by regulating

gene expression. Tools for silencing genes include antisense oligonucleotides, ribozymes, siRNAs, etc. For example, siRNAs is a common gene therapy drug that mainly combines with complementary mRNA molecules to inhibit 35 gene expression and protein synthesis. However, microRNAs are extremely fragile, easily degraded by enzymes, and have a short half-life in vivo. Additionally, there are also problems related to toxicity, immunity, or inflammation, making them unsuitable for clinical application. As a biocompatible carrier, the DNA frame materials have attracted much attention in gene silencing by carrying antisense DNA or siRNA. Many studies have proved that constructing a new siRNA delivery system using DNA framework materials can effectively silence target genes in tumor cells. The same principle is also applicable in antibacterial research. Aptamers are single-stranded RNA or DNA sequences with selective recognition function. Compared with antibody, aptamer has a flexible design, convenient synthesis, modification, low cost, and good biochemical stability. Therefore, in recent years, aptamers have been widely used in biological analysis and sensing [22, 23]. Researchers discovered a small bacterial regulatory RNA in 50–500 nucleotide length bacteria and proved that it has a specific effect on antibiotic resistance treatment [24]. Small RNA (sRNAs), also known as regulatory sRNAs, can be divided into CIS and trans according to the base-pairing algorithm. Multiple studies have shown that sRNAs play an essential role in controlling bacterial gene expression induced by extracellular stress and maintaining the stability of the intracellular environment of microorganisms [25–28]. SRNAs are expressed during the bacterial transformation from colonization to active infection [29, 30], which is also one of the organism's mechanisms to adapt to environmental changes. Figure 6.2 shows the association between small bacterial RNAs and antimicrobial resistance pathways. Exposure to antibiotics is an environmental stimulus that contributes to the physiological changes of bacterial cells. According to the results, sRNAs' expression was different after antibiotic exposure [31–35]. The regulation of the sRNAs sensitivity to antibiotics is related to the

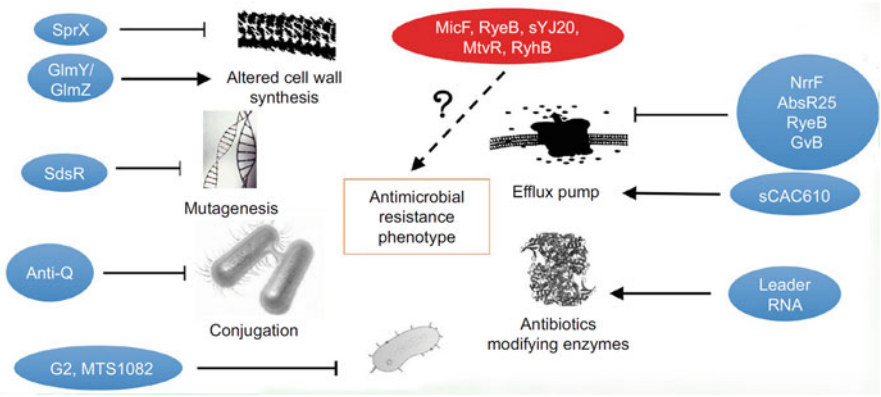

Fig. 6.2 Small bacterial RNAs interact with canonical (in blue) and unknown mechanisms (in red) in acquisition of antimicrobial resistance phenotype. The pointed arrowheads denote putative stimulatory effect, whereas the blunted arrowheads represent inhibitory actions. Copyright 2017, Infection and Drug Resistance [23]

synthesis of the cell wall, outer membrane protein, efflux pump, and transporter. Nonspecific sRNAs do not express high specific mRNA, while specific sRNAs interact with multiple mRNA targets [36, 37]. The researchers also discovered that Hfq and CSRA reduced bacterial infection and increased bacterial sensitivity to antibiotics. The reason could be that it suppresses many trans RNAs' activity and then suppresses the downstream sRNAs [38, 39]. Recombinant sRNAs is also a method to increase the sensitivity of bacteria to antibiotics. T. Kim and other scholars found that following exposure to ciprofloxacin, gentamicin, tobramycin, tetracycline, ampicillin, chloramphenicol, and the upregulated expression of MTVR significantly inhibited the growth of the *Pseudomonas aeruginosa* [40]. On the contrary, the regulation of antibiotic sensitivity induced by sRNAs is reversible through the absence of corresponding sRNAs [41]. Kangyk et al. used the CRISPR-cas9 system as a carrier and then complexed it with single RNA for antibiotic resistance. sgRNAs were designed to target a specific sequence within the mecA gene. BPEI covalently modified the endonuclease of cas9. It can form small nanocomposites when mixed with sgRNA. Their results showed that the nanocomposites targeting MECA, a major gene related to methicillin resistance, could be effectively delivered to *MRSA* to achieve nonviral and therapeutic genome editing and may be used as targeted specific antibacterial drugs [42]. Unlike traditional antibiotics, sRNAs are an influential gene regulatory factor in regulating genes by inhibiting bacterial protein synthesis translation. As a new drug or auxiliary component, sRNAs have great application potential. Besides, considering that a single sRNAs molecule has multiple mRNA targets, using phages or nanoparticles to carry sRNAs in vivo is an effective transportation method. Comprehensive studies on the in vitro experiments of clinical isolates and in vivo experiments of animal models are needed to elucidate the clinical status and therapeutic potential of sRNAs.

6.3 Antisense Oligonucleotide (ASODN) and Antisense Peptide Nucleic Acid (PNA)

Antisense therapy is a type of biotechnological treatment that uses the chemical analogs of short single-stranded nucleic acid sequences to modify into stable oligomers [43]. Antisense antimicrobial substance is a synthetic DNA analogue with about 10–20 bases that can inhibit the expression of specific genes in a particular sequence at mRNA level [44, 45]. Over the past few years, the stability of various modified ASODNs in biological media and their ability to specifically bind to target RNA has been confirmed [46, 47]. ASODN modifies the gene expression by binding to complementary mRNA and inhibiting its transformation to protein by spatial blocking or degradation of ASO/RNA double-stranded RNase. Depending on the target gene's function, ASODN may have either bactericidal or bacteriostatic effects and may also have the functionality of restoring bacterial susceptibility. Several studies have shown that they are specific for mRNA targets and can inhibit translation [48–61]. N. Nekhotiaeva et al. first presented evidence of genes necessary for the growth of

Staphylococcus aureus. Hmrb is a homologous gene of acpp gene, which is highly sensitive to antisense inhibition of *Escherichia coli* (*E. coli)* PhoB is the gene encoding alkaline phosphatase. GyrA is involved in DNA replication, and fmhb is involved in cell wall biosynthesis. The study demonstrated the importance of these genes by testing the corresponding PNA's bacteriostatic [58, 62]. Free nucleic acids cannot spontaneously pass through the cell wall and membrane of bacteria, so a carrier is needed to aid them in entering the bacteria. Federico Perche et al. proposed a novel liposome polymerized skeleton nucleic acid system that can transfer nucleic acids with specific sequences to bacterial cells [63]. Its nucleic acid consists of 11 nt antisense single-stranded DNA, 15 bp and 95 bp double-stranded DNA, 9kbp plasmid DNA, and 1000NT single-stranded RNA. Flow cytometry showed that *E. coli* and *Pseudomonas aeruginosa* could effectively internalize these compounds. An antisense oligonucleotide primarily inhibits the antibacterial effect of this kind of nucleic acid skeleton structure. It can induce specific and important bacterial growth genes to be inhibited by targeting an essential gene, leading to a bactericidal effect. The high efficiency, versatility, and broad-spectrum activity of ASODN provide strong support for the potential application of DNA nanostructures combined with nucleic acids in bacterial infection treatment. The application of antisense technology in bacterial microbial therapy is still facing significant challenges. Maintaining in vivo activity, reducing the biological toxicity of ASODN, and enhancing the pharmacokinetic behavior of ASODN are yet to be solved. However, they may inhibit the growth of specific microorganisms in complex microbial communities. In the future, it is expected that the chemical modification of ASODN will further improve the effectiveness and specificity of ASODN targeting. It is usable in clinical and nonclinical biotechnology fields, such as complex microbial communities in industry and the environment. Recent research on ASODN or PNA against bacteria is shown in Table 6.1.

6.4 Combined Application of DNA Nanomaterials and Antisense Technology

The main barrier for ASODN or PNA to enter bacteria is the bacteria's cell wall and the thick layer of bacterial exopeptidoglycan. Consequently, ASODN or PNA requires an effective means of transportation. Recently, the coupling of synthetic antisense molecules with cell-penetrating peptide (CPP) has become a practically effective bacterial drug delivery pathway [51, 64]. However, CPP is limited to antisense modification without a positive charge; otherwise, it will form a negative deposit. These conjugates only showed the effect in a relatively high concentration bacterial infection model [65]. On the other hand, CPP's strong cytotoxicity limits its development in vivo [66, 67]. Therefore, treatment research should aim to develop a safer and effective drug delivery system. A good drug delivery system should effectively package ASODN to prevent its degradation. Also, it cannot induce an immunogenic response in the host [68]. The synthesis of CPP coupled ASODN or PNA entails an expensive and time-consuming process. In addition, some studies

Table 6.1 Researches on phosphorothioate oligonucleotide (PSODN) or PNA in recent years

Reference	PSODN or PNA	Sequence (N′-C′) / (5′-3′)	mRNA	Carrier	Organism	MIC
Kurupati et al. [48]	ompA	(KFF)3 K-ATACCAGGTGTTATCT	ompA	No	*K. pneumoniae*	40 μM
Dryselius et al. [49]	Ec109	(KFF)3 K-GTCATGTTTT	fabD	No	*E. coli*	2.5 μM
Liang et al. [50]	PPNA1	(RXR)4XB-eg1-CATTTAAATTTC	ftsZ	CPP	*MRSA*	40 μM
Good et al. [51]	Spp4	(KFF)3 K-CTCATACTCT	acpP	No	*E. coli*	0.6 μM
Nekhotiaeva et al. [52]	Sau101	(KFF)3 K-CCATGATTTA	fmhB	No	*S. aureus*	10 μM
Rajasekaran et al. [53]	polA	(KFF)3 K-TTCATGCCTGT	polA	No	*B. suis*	10 μM
Kulyté et al. [54]	Ms101	(KFF)3 K-GTCATTTGGT	inhA	No	*M. Smegmatis*	<5 μM
Patel et al. [55]	acpP-CPP1	(KFF)3 K-CTCATACTAT	acpP	No	*E. Amylovora*	2.5 μM
Meng et al. [56]	PS-833	CGAGTCCCTTTTTACCAA	mecA	PEI	*S. aureus*	8 μM
Harth et al. [57]	HP2/ HP1	GCGCATATGCGCAATCTTTCGGCTCACGTCTGTCATGCGCGCGC	Mycolyl transferase	Hairpin extensions	*M. Tuberculosis*	10 μM
Bai et al. [58]	rpoD	TTTGCTCCAT	rpoD	(RXR) 4XB	*S. Flexneri*	5 μM
Chen et al. [59]	rpoD	N/A	rpoD	N/A	*MRSA*	N/A

(continued)

Table 6.1 (continued)

Reference	PSODN or PNA	Sequence (N′-C′) / (5′-3′)	mRNA	Carrier	Organism	MIC
Hegarty et al. [60]	rpoB	CATTCACTTTTCACCTCTCAATAAT	rpoB	Bolasome complex	*C. difficile*	0.2 μM

(Abbreviations: *K. pneumoniae, Klebsiella pneumoniae; MRSA, methicillin-resistant Staphylococcus aureus; E. coli, Escherichia coli; S. aureus, Staphylococcus aureus; B. suis, Brucella suis; M. smegmatis, Mycobacterium smegmatis; E. amylovora, Erwinia amylovora; PEI, polyetherimide; M. tuberculosis, mycobacterium tuberculosis; S. flexneri, Shigella flexneri; C. difficile, Clostridium difficile*; NA, data not available.)

have shown that bacteria are also resistant to CPP, which could be related to the gene mutation encoding the active transporter SBMA [69]. The research group synthesized the nucleic acid frame material with a tetrahedral structure based on ssDNA's three-dimensional structure. It can penetrate the bacterial cell wall and integrate PNA into its structural design, thus providing active and directional PNA synthesis [70, 71]. Tetrahedral DNA nanostructures have the advantages of low cost, fast assembly speed, and simple operation, which are essential carriers for ASO preparation [72]. Ana Gonzalez Paredes et al. studied the antimicrobial ASODN transcription factor decoy (TFD). This short double-stranded DNA molecule can capture vital regulatory proteins blocking necessary bacterial genes and defeating infections [73]. The authors considered the iron uptake pathway of *E. coli* as the target and verified the antibacterial effect of TFD under the condition of micro-oxygen. Readman J.B. and others developed a self-assembled biocompatible DNA tetrahedral nanoparticles carrier to restore the antibiotic sensitivity of cefotaxime resistant *E. coli*. A targeted antisense peptide nucleic acid was added to its structure to penetrate the bacterial cell wall [70]. Biofilm formation can lead to chronic infection. Bacteria and extracellular polysaccharides (EPS) cause biofilm adhesion. In addition to being toxic, they are also resistant to antibiotics. The inhibition of EPS synthesis can prevent the formation of bacterial biofilm. Here, the author developed a tetrahedral frame nucleic acid delivery system. It can transmit antisense oligonucleotides to specific genes. Once they enter bacterial cells, they can significantly reduce EPS synthesis and the thickness of biofilm. Concurrently, it reduced the expression of all target genes (gtfbcd, GbpB, FTF), which had high efficiency. In general, they have great potential in treating chronic infections caused by biofilm [74].

6.5 Combination of DNA Nanostructures and Antibiotics

Table 6.2 summarizes the bactericidal mechanism, resistance mechanism, and related genes and proteins of several common antibiotics [75–83]. The use of drug delivery systems is a practical approach to increase the lethality of antibiotics. Presently, nucleic acid nanomaterials have become an indispensable part of the drug delivery system, which includes DNA positive bodies that can be used to deliver nucleic acid drugs and small molecule drugs [84]. The combination of DNA nanostructures and antibiotics optimizes antibiotics' bactericidal performance, reduces the toxicity of antibiotics to human cells, and reduces the demand for high-dose antibiotics [85, 86]. Several studies have shown that the combination of antibiotics and DNA nanostructures can improve antibiotic concentration at the bacterial antibiotic interaction site and promote the affinity of antibiotics to bacterial cells. Likewise, synergistic effects can be observable when nanoparticles combine with essential oils or antimicrobial peptides. Researchers constructed a novel antibacterial and diagnostic composite antibiotic nanostructure using self-assembled DNA nanoparticles (DP) as scaffolds to detect and treat bacterial infection [87]. Setyawati et al. prepared a teragnostic DNA nanostructure containing diagnostic auncs and therapeutic actinomycin D (AMD), named dpau/AMD [88]. AMD is

Table 6.2 The antibacterial mechanism, resistance mechanism and related genes and proteins of common antibiotics

Types of antibiotics	Antibacterial mechanism	Drug resistance mechanism	Resistance related genes/proteins
Tetracycline	Tetracyclines preferentially bind to bacterial ribosomes and interact with highly conserved 16SrRNA targets in 30S ribosomal subunits to form reversible complexes. In the process of extension, they inhibit the synthesis of proteins necessary for bacterial growth and survival by spatially interfering with the binding of charged aminoacyl tRNA to mRNA ribosome complexes, and preventing new amino acids from entering the new peptide chain	1. The efflux pump gene encodes membrane related efflux proteins, which can pump drugs out of the cell actively, leading to the decrease of drug concentration in the cell 2. (Ribosomal protection proteins, RPPS) combined with ribosome can reverse the distortion of ribosome structure, cause the change of ribosome configuration, directly interfere with the interaction of tetracycline D-ring and 16SrRNA base c1054, so that tetracycline drugs cannot be combined with it and dissociate from the 30S subunit of the binding site, so as to protect ribosome	1. The most common tetracycline specific efflux pump is a member of the major transporter superfamily (MFS) 2. There are 13 tetracycline resistance genes in *E. coli*: 9 tetracycline efflux genes tet (a), tet (b), tet (c), tet (d), tet (E), tet (G), tet (J), tet (L) and Tet (y), respectively
Macrolides	It can irreversibly bind to the 50S subunit of bacterial ribosome, resulting in the ribosome bound with macrolide unable to polymerize the specific amino acid sequence in the new protein, thus blocking the process of peptide transfer and mRNA transfer, thus blocking the growth of peptide chain, inhibiting the synthesis of protein, and finally playing a bacteriostatic role	1. The N6 position in nucleotide A2058 is monomethylated or dimethylated. Methylation can interfere with the formation of hydrogen bonds, resulting in a significant decrease in the affinity between macrolides and ribosomal 50s subunits, resulting in the production of resistant strains 2. Reduce intracellular concentration by using efflux pump	1. Erythromycin resistant methyltransferases (ERMS) gene encoding methyltransferase 2. MSR protein provides ribosome protection by binding macrolides. MEF (a) and MEF (E) are the most common, which can lead to bacterial resistance to 14, 15 membered macrolides
β-lactams	The inhibition of transpeptidase may lead to the inhibition of bacterial cell wall synthesis	1. The β-lactamase produced by bacteria can covalently combine with the carbonyl part	1. Mutations of PBPs 2. Overexpression of MEXA or b-oprm is one of the main

(continued)

Table 6.2 (continued)

Types of antibiotics	Antibacterial mechanism	Drug resistance mechanism	Resistance related genes/proteins
	and bacterial death. Penicillin binding proteins (PBPs) are the main targets of β-lactam antibiotics	of antibiotics and destroy its ring structure, resulting in the degradation of β-lactamases before reaching the target 2. The loss of affinity between β-lactam antibiotics and their target PBPs, the drug cannot play its role by binding with its action site, which leads to bacterial resistance 3. The change of cell membrane permeability or the increase of efflux pump activity will make antibiotics unable to enter the bacteria, which will reduce the combination of drugs and targets, thus reducing the activity concentration of antibiotics in the bacteria	reasons for drug resistance of *Pseudomonas aeruginosa* and other pathogenic gram-negative bacteria
Aminoglycosides	Protein synthesis was inhibited by a site on the 16SrRNA decoding region of 30S ribosomal subunit with high affinity	1. The decrease of membrane permeability may lead to the decrease of drug uptake and accumulation of bacteria, which may lead to drug resistance 2. Bacteria can produce aminoglycoside modifying enzymes (Ames), inactivate aminoglycoside antibiotics, and lead to antibiotic resistance 3. The change of the target, the antibiotic cannot combine with ribosome	OmpF in *Escherichia coli* and OPRD in *Pseudomonas aeruginosa* act as nonspecific entry and exit points in antibiotics and other small molecular organic chemicals
Quinolones	By inhibiting DNA helicase and topoisomerase, it can interfere with the process of DNA replication and	1. It is QRDR. In this region, mutations are most common at codon 83 and 87. The mutation of the active site	1. The first is the mutation of gyrA and gyrB encoded a and B subunits in DNA gyrase

(continued)

Table 6.2 (continued)

Types of antibiotics	Antibacterial mechanism	Drug resistance mechanism	Resistance related genes/proteins
	transcription to achieve antibacterial effect	may change the binding of quinolones to the site, thus reducing the sensitivity to quinolones 2. These plasmid mediated mechanisms include qnr like proteins that protect DNA from quinolone binding, modification of some AAC (6 ')-ibcr acetyltransferases, and active efflux pump proteins	2. The second is the mutation of C and e subunits encoded by Parc and pare, respectively, in topoisomerase IV
Sulfonamides	By interfering with the synthesis of folate in bacteria	Resistance is achieved by producing low affinity dihydrofolate synthetase	Sul1, sul2 and sul3 can increase the expression of this resistant enzyme

used as an antimicrobial agent, while bacterial detection uses red emission glutathione protected gold nanoclusters. According to the results, *E. coli* and *Staphylococcus aureus* were more easily ingested in the platform of thermal sensitivity—dpau/AMD—and bacteria were more sensitive to antibiotics in the platform than in the free AMD. The effective killing effect of dpau/AMD on infectious bacteria could be due to the degradation of DP structure by DNase, which releases AMD. The most common way for bacteria to produce antibiotic resistance is to produce an efficient enzyme, called β-lactamase, which catalyzes the β-lactamase ring's breaking. Such an enzyme inhibitor can be used in combination with the existing β-lactam antibiotics. However, until now, there has been no β-lactamase inhibitor available in clinical settings [89, 90]. Xiangyuan Ouyang et al. constructed a DNA nanoribbon with a width of 16 nm and found that it was a novel and broad-spectrum inhibitor of the β-lactamase. The authors used a DNA nanostructure inhibitor targeting the clinically relevant metallo-β-lactamase. Their discoveries provided a new platform to design macromolecular inhibitors combined with β-lactam antibiotics against multidrug-resistant bacteria [91]. DNA naturally exists in every living cell; as such, it has a high degree of biocompatibility. It is loadable with antibacterial drugs without adverse effects on the body's cells [92]. In general, the combination of DNA nanostructures and antibiotics indicates another promising research direction in the future. However, the study of DNA tissue engineering is still in the initial phase. There will be many problems and challenges in the field of drug delivery research. For example, the cell environment is complicated, containing a significant number of enzymes, nucleic acids, proteins, and other molecules. The drug delivery system of DNA space materials will encounter many types of enzyme degradation and nontarget biomolecule interference. Besides, how to further improve the

stability, sensitivity, and accuracy of materials remains a challenge. On the other hand, most of these studies focus on the transfer of nucleic acids or small molecules. In contrast, a few studies focus on the spatial structure of DNA tetrahedron and intelligent drug release control. Given the rapid development of DNA framework materials and DNA nanotechnology, there is an optimistic feeling that these challenges could be overcome in the near future.

6.6 Combined Application of DNA Nanostructure and Metal Nanoparticles

The inhibition of enzyme activity and the efflux pump's effectiveness form the primary defense ways for bacterial cells to diminish the sensitivity to antibiotics. In cells, both metal ions and nanoparticles induce the production of ROS. Metal nanoparticles can effectively bind to the surface of bacteria and destroy their cell walls, which leads to cell death. Moreover, nanoparticles can release metal ions from extracellular space, which can enter cells and disrupt biological processes. The researchers identified that metal nanoparticles smaller than 20 nm could penetrate the bacterial cell wall and cause bacterial death by destroying the organelles. According to scientists' research, silver is the most effective antibacterial agent among all kinds of metal nanoparticles. Other antibacterial metal nanoparticles include the antibacterial activities of iron, zinc, and gold. The antibacterial activity of silver nanoparticles depends on their size and shape. The decrease of the size and the increase of silver nanoparticles' surface area lead to the enhancement of the binding affinity with molecules. Compared with spherical or rod-shaped silver nanoparticles, triangular silver NPs exhibited more significant antibacterial activity [93]. AG NP can produce living oxygen, oxidize DNA, and protein in bacteria, leading to metabolism and cell division failure. AG NPs affect the signal transduction of *E. coli* cells through the change of tyrosine phosphorylation. In addition, AG NPs also affect the formation of biofilm by preventing the growth of biofilm. Based on the results, the pernicious effect of Ag NPs on gram-negative bacteria was better than that on gram-positive bacteria. In addition to a thin layer of peptidoglycan, some phospholipopolysaccharides (LPS) are also present in the phospholipid outer membrane of gram-negative bacteria, which increases the negative surface charge of their cell membrane. Due to the electrostatic interaction, the negatively charged bacterial cell wall attracts the positively charged nanoparticles to its surface. Meanwhile, the positively charged metal nanoparticles share a strong bond with the membrane, which leads to the rupturing of the cell wall, thus increasing their permeability. When silver ions combine with gram-negative bacteria, holes are formed on the cell wall so Ag NP can penetrate the cell [94, 95]. Numerous studies have discovered that iron nanoparticles extracted from plants have apparent inhibitory effects on *Staphylococcus aureus*, *Enterobacter*, *Staphylococcus mirabilis*, and *E. coli* [96]. Zinc oxide has the function of photooxidation and photocatalysis, and it is also considered as biosafety in metal nanoparticles. Pathogenic bacteria have cell surface proteins for

adhesion and colony formation. Polysaccharides and cholic acid also exist on the cell wall to protect it from the defense mechanism of the host and environmental conditions. Since these are charged macromolecules, surface modified nuclear power sources can effectively induce specific interactions, thus destroying the integrity of the cell walls. Zinc oxide nanoparticles interact directly with bacterial cell walls, destroying their integrity. Zinc oxide has a strong absorption of ultraviolet light. Zinc oxide NP has a pronounced phototoxic effect on bacterial fermentation broth. Obviously, ROS can be observed when zinc oxide NP is used to treat bacterial fermentation broth and ultraviolet irradiation bacterial fermentation broth [97]. Gold nanoparticles have good biocompatibility and antibacterial activity. Au NPs cannot act on the target alone, so it must be labeled with other biomolecules to inflict effective antibacterial performance. AuNPs can be combined with antibiotics to observe the synergistic antibacterial effect. Gold nanoparticles could enter cells and change the membrane potential by inhibiting ATPase activity, which leads to energy metabolism collapse and cell death. This non-ROS-dependent pathway can also kill MDR bacteria. Similarly, size also has a significant influence; smaller AuNP shows a better bactericidal effect than a larger one [98].

In general, the principle of metal nanoparticles in antibacterial has four points [99–102]: (1) ROS's antibacterial effect: ROS (such as superoxide anion, hydroxyl radical, and hydrogen peroxide) is produced after exposure to metal oxide and other nanomaterials. These reactive oxygen species induce DNA damage by peroxidation of polyunsaturated phospholipids in bacterial cells, leading to cell death. (2) The antibacterial effect of physical damage: the bacterial cell wall membrane may be damaged when interacting with nanostructured materials' sharp edge. (3) Antibacterial effect of binding: the binding materials on the bacterial cell wall could lead to the loss of cell membrane integrity and the outflow of cytoplasmic materials. (4) Antibacterial effect by releasing metal ions: metal ions released from nanomaterials to culture medium could inhibit the production of ATP and DNA replication, thus damaging cells. The collective application of metal nanoparticles and DNA nanostructures focuses on the formation of various structural sensors, such as microRNA, mRNA, protein, small molecule, and DNA [103–106]. Figure 6.3 shows the antimicrobial mechanisms of metal ions which are antimicrobial. The antibacterial properties of nanogold, nano silver, nano zinc, and other metal nanoparticles have been studied extensively. Microbial experts agree that it has an antibacterial effect in vitro. However, metal nanoparticles are toxic, and humans cannot metabolize them, so there are still significant challenges in the practical clinical application of antibacterial. Evidently, the combination of DNA nanostructures and metal nanoparticles can improve antibacterial drugs' bioavailability and reduce the occurrence of drug resistance. The polymer formed by nanoparticles has potential antibacterial activity, which could further enhance antibacterial particles' efficacy [107–109]. As a single antibacterial drug, silver is limited because of its potent toxicity to host cells. Some scholars use aptamers to target Ag nanoclusters to *Pseudomonas aeruginosa* to achieve the purpose of anti-infection. The invertebrate infection model was used to evaluate the antibacterial activity in plankton culture and in vivo. According to the outcomes, compared with the same number of nontargeted silver nanoclusters, the targeted silver nanoclusters

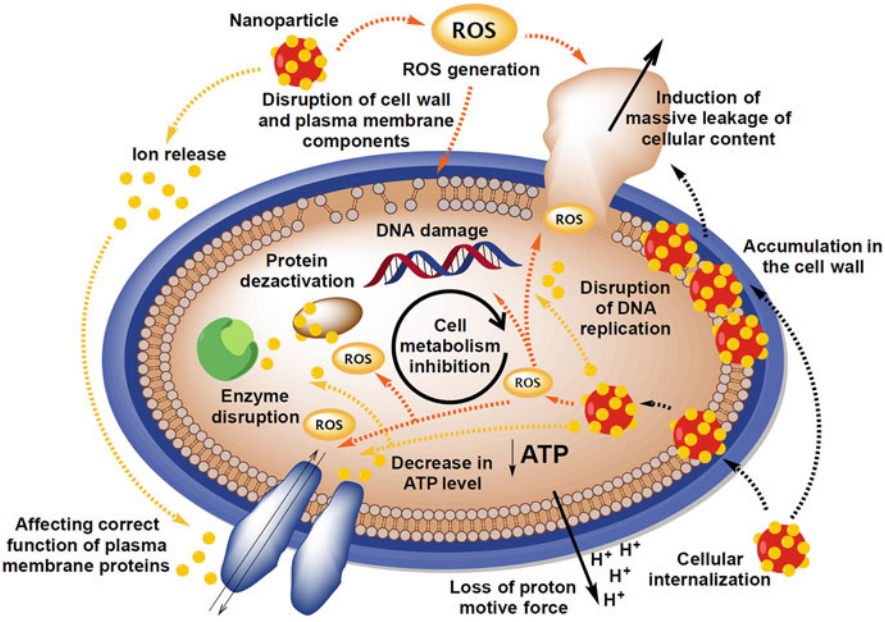

Fig. 6.3 Schematic representation of antimicrobial mechanisms of metal ions. Copyright 2016, Elsevier [112]

had a better killing effect on *Pseudomonas aeruginosa* [110]. In vitro and in vivo animal models, it has been proved that DNA aptamer targeting silver nanoclusters is practical with rapid antibacterial activity. And it dramatically reduces the required dose, thus decreasing the biological toxicity. I. Armentano et al. reviewed that the DNA chain covered by nano silver particles is the key to stabilize the antibacterial activity [111]. After evaluating several DNA strands, it was found that the antibacterial activity of nano silver depended on the DNA sequence used in its preparation. Some scholars use circular dichroism to analyze DNA nanomaterials' structure and have found that the derivatives measured have significant differences in antibacterial properties, which indicates that the morphology and structure of DNA nanomaterials could be the key to their antibacterial activities. Surprisingly, these DNA AgNPs exhibit extremely low toxicity in addition to the decent antibacterial effect, which is a promising compound for in vivo application.

6.7 Combined Application of DNA Nanostructure and Antimicrobial Peptide (AMP)

Antimicrobial peptides (AMPS) are small peptides widely existing in natural organisms; they are essential components of the innate immune system. Antimicrobial peptides have a wide range of inhibitory effects on bacteria, fungi, parasites, viruses, tumor cells, etc., which provides them a good prospect in the pharmaceutical industry, food additives, and other fields. The antibacterial functions of antimicrobial peptides include anti-gram-positive bacteria, gram-negative bacteria, and gram-concurrent bacteria [113]. Antimicrobial peptides can interact with the surface of the cell membrane and alter the permeability of the membrane. The interaction between the positively charged area and the negatively charged area on the cell membrane makes the hydrophobic end of the antibacterial peptide molecule insert into the cell membrane's lipid membrane. It then changes the structure of the lipid membrane. Following antibacterial peptide and cell membrane action, it forms transmembrane potential, breaks acid-base balance, affects osmotic pressure balance, and inhibits respiration [114]. Besides acting on the cell membrane, antimicrobial peptides can also act on other targets in the cell. After entering the cell, the antibacterial peptide interferes with the cell metabolism through the specific combination with the cell's target to inhibit and kill the bacteria. Under this theory, many antimicrobial peptides can still cause bacterial death at low concentrations. Antimicrobial peptides play an essential role in cells through the following aspects: (1) binding with acid substances in cells to block DNA replication and RNA synthesis, (2) affecting protein synthesis, (3) inhibiting cell wall synthesis and cell division, and (4) inhibiting enzyme activity in cells, etc. [115]. There are currently more than 60 peptide drugs on the market, and hundreds of new therapeutic peptides are in the preclinical and clinical development stages. The physical and chemical properties of existing peptides can be further improved through practical design. The low stability, high toxicity, and increased application cost of natural antimicrobial peptides hinder its clinical application. The next step is to achieve amp stability in various environments to use these molecules as vaccines or ointments. Effective delivery methods overcome rapid degradation and removal. Different functionalization may change AMP's selectivity and biological activity, which will result in the rapid degradation or elimination of antimicrobial peptides [116]. The low penetration and instability of amps to mammalian cells in vivo limit its application in intracellular pathogens treatment [117]. Apart from increasing the antibacterial activity, the newly synthesized amps are also designed to reconfigure peptides to achieve higher penetration, selectivity, and anti-degradation and reduce hemolytic activity or cytotoxicity to healthy cells. AMPS' design and eventual application as a frontier and challenge for new therapies include obtaining precise control of these functions. Specificity is another prominent feature that people are increasingly considering, because the next generation of antimicrobial agents should be designed to kill selected pathogens without damaging the organisms that make up the host-microbiota [118]. Jin Hyun Yeom and other researchers found that gold

nanoparticles combined with DNA aptamers can effectively transfer amps to mammalian cells. In addition to enhancing the stability of AMPS, it also enhances its effectiveness [119]. AuNPs and amps were mixed in a relatively straightforward way to form AuNPs APMS. These were injected into HeLa cells of *Salmonella* serotype to treat HeLa cells of *Salmonella* serotype. Their results showed that AuNP APMS eliminated typhimurium cells and improved the viability of host cells. Additionally, the study also conducted in vivo studies in mouse models by injecting their AuNP APMS through the caudal trunk line into mice infected with *S. typhus*. The results showed that the colonization of *Streptococcus macularis* in mouse organs was inhibited entirely, and 100% of the mice survived. Based on the existing research data, we believe that this combination may become a candidate for further study of drug-resistant bacteria treatment. The present research shows that antisense peptide nucleic acids are transferred to MRSA cells via noncytotoxic tetrahedral skeleton nucleic acids (tFNAs) as delivery carriers. The expression of FtsZ was successfully inhibited by the effective transport of tFNAs to specific gene FtsZ. This study also uses tFNAs as the carrier to deliver ampicillin to study whether it has a decent antibacterial effect on drug-resistant *staphylococcus aureus*. Therefore, the combination of DNA nanostructures and antimicrobial peptides can be used as an innovative platform for the treatment of bacterial infection in mammalian cells [120, 121].

6.8 Antimicrobial Studies of Some Other Nucleic Acid or Analogues

F. Nassar et al. developed a novel uracil derivative and tested its antibacterial, antioxidant, and anticancer activities. The analysis found that the material was more effective against gram-positive bacteria than the control drug cefoperazone. It also has high antibacterial activity against gram-negative bacteria [122].

Topoisomerase is an enzyme existing in the nucleus, which catalyzes the breaking and binding of DNA strands. It controls the topological state of DNA and plays an essential role in the organism. Generally, type II topoisomerase inhibitors are DNA rotatase and type IV topoisomerase double target inhibitors, which can play an antibacterial role by blocking their ATP binding sites or catalytic processes. Tari et al. found tricyclic compounds with pyrimidine and indole as the core; these could bind to ATP binding sites of DNA gyrase and type IV topoisomerase to assume an antibacterial role [123]. Topoisomerase DNA covalent complexes can be used as drug targets of novel topoisomerase inhibitors, representing a new antibacterial drug class. P. B. Tiwari et al. studied the molecular characteristics of the critical function of *E. coli* topoisomerase I (exopoi)-DNA covalent complex (exopoi CC). The work laid a foundation for the development of new antibacterial drugs. The researchers tested the direct binding of nsc76027 to heterotopic sites and the inhibition of their relaxation activity through experimental techniques. A molecular dynamics simulation was conducted to study the dynamic behavior of ternary complexes. The

simulation results show that nsc76027 forms a stable ternary complex with an external point. The external point studied here is also usable as a model system for studying topoisomerase and DNA complex, where DNA covalently connects to protein. The transient covalent complexes of DNA topoi and DNA were captured in bacterial cells by topoi, which led to the accumulation of these complexes and ultimately killed bacterial cells.

6.9 Conclusion

Since DNA has been developed into nano scale self-assembly materials, it is related to two fields of life science and material science: adjustable multifunction, convenient programmability, accurate molecular recognition ability, and high-throughput, superior biocompatibility, and biodegradability. Owing to the structural characteristics of DNA nanomaterials, functional nucleic acid DNA materials, which are cross-linked and self-assembled, DNA nanomaterials have become a research hotspot in the field of new materials. Compared with the study of nucleic acids in eukaryotic cells, there are lesser studies on bacterial. The development of nucleic acid materials provides a unique choice for antibacterial therapy. The existing analysis focuses on using aptamers or skeleton nucleic acid structures as drug carriers to improve the local concentration of antibiotics and enhance their affinity to bacteria. Some studies have been looking for ways to kill drug-resistant bacteria directly to reduce the MIC of antibiotics at the gene level. The use of nucleic acid technology can inhibit or upregulate the expression of specific genes, leading to bacterial growth and metabolism in the process of being blocked and dying. The team working on this research is also working in this field to provide more reliable scientific evidence for the effectiveness of nucleic acid materials in antibacterial applications.

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Chapter 7
The Application of DNA Nanostructures in Vaccine Technology

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Abstract Vaccine is a biological agent for preventing and curing disease, which inducts both innate and adaptive immune mechanism to be effective. Facing potentially unknown pathogens, the current vaccine technologies have problems such as (1) prolonged development time, (2) limited production capacity, and (3) inability to guarantee biosafety. To address these issues, DNA nanostructures as carrier platforms, featured with strong immunogenicity, excellent biosecurity, and promising programmability, have attracted much attention in the development of vaccines nowadays. These DNA nanostructures, including DNA tetrahedra, DNA hydrogel, DNA nanotube, DNA dendrimer, and DNA nanoflower, could not only directly induce macrophages to secrete immune factors by modifying sizes and structures but also indirectly stimulate TLR9 immune response as carriers of CpG ODNs. In addition, DNA sequences can be combined with different antigen molecules to form an antigen presentation system to participate in the body's adaptive immune response. This review summarizes the role of various DNA nanomaterials in the field of immunity and aims to provide new ideas for enhancing the body's immune response against diseases and treating various immune system diseases.

Keywords Vaccine · DNA nanomaterials · Immune response · Immunoadjuvant

Abbreviations

anti-dsDNA	anti-double-stranded DNA
APC	Antigen-presenting cells
ASCs	Antibody-secreting cells
BSA	Bovine serum albumin
BS-nanow	Bead-chain DNA nanowires

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CFA	Complete Freund's adjuvant
cGAMP	Cyclic GMP-AMP
CpG	Cytosine-phosphate-guanine
CTL	Cytotoxic T lymphocyte
DL-DNA	Dendrimer DNA
DNase I	Degradation of endonuclease
DNO	DNA nano-octahedron
DOX	Doxorubicin
DSHV	DNA supramolecular hydrogel vaccine
dsODN	Double-stranded ODN
E-DNO	Encapsulated DNO
ELISPOT	Enzyme-linked immunosorbent spot
FDG	18F-fludeoxyglucose
iDR-NC	DNA-RNA nanocapsules
IFN	type I interferon
INH-ODN	Immunosuppressed oligodeoxynucleotides
IRF3	Interferon-regulatory factor 3
JAK/STAT	Janus kinase/signal transduction and transcription activation
JNK/SAPK	Jun N-terminal protein kinase/stress-activated protein kinase
KK	KK1B10
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MYD88	Myeloid differentiation primary response 88
N-DNO	Nonencapsulated DNO
NF-κB	Nuclear factor-kB
OVA	Ovalbumin
pDC	Plasma cell-like DC
PLG	Polymeric nanomaterials including poly (d, l-lactide-co-glycolide)
PLGA	Poly(d, liter-lactic acid-hydroxy acid)
RCR	Rolling cycles
RGC	Retinal ganglion cells
ROS	Reactive oxygen species
shRNA	Short hairpin RNA
ssODN	Single-stranded ODN
STINGs	Stimulator of interferon genes
STV	Streptavidin
TDN	DNA tetrahedron
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
VLPs	Virus or virus-like particles
VLPs	Virus-like particles
Y-DNA	Y-type DNA
Y-ODNs	Y-shaped oligodeoxynucleotides

7.1 Introduction

Ever since the invention of the first vaccine, vaccination have helped save many lives and significantly improved the quality of life. As the most effective medical intervention to control or even eliminate a disease, vaccination can be considered as one of the greatest breakthroughs in modern medicine [1, 2]. Like nature infections, vaccines act by initiating both innate immune and adaptive immune response [3]. Innate immunity occurs within hours of pathogen recognition, followed by an adaptive immune response over several days, leading to immune memory [4]. Currently, live attenuated vaccines usually produce an effective and durable immune response. However, in the case of inactivated vaccines, adjuvants are often required to enhance the efficacy of antigen. Therefore, researches on vaccines in recent years have focused on adjuvants which enhanced the activity of vaccine delivery systems. Adjuvants can be broadly divided into three types of delivery systems: immunomodulatory molecules, non-immunostimulating component antigen delivery systems, and delivery systems that have both functions [5].

The most widely used immunomodulatory molecule in the field of immunity is cytosine-phosphate-guanine (CpG) oligonucleotide. It can activate the myeloid differentiation primary response 88 (MYD88) signaling pathway by interacting with the host's own CpG DNA, through which type I interferon (IFN) and other pro-inflammatory cytokines can be produced [6]. In addition, some clinical trials in humans to evaluate the activity of CpG ODN adjuvants showed that CpG ODNs can induce a T1 immune response and become potential cancer vaccine adjuvants [7]. Among different types of CpG ODNs, D-type ODN can effectively induce plasma cell-like DC (pDC) to produce type I interferon, but cannot activate B cells to produce antibodies. Due to the presence of multiple G tails, D-type ODNs may form aggregates, which limits their applications. K-type CpG ODN (or B-type ODN) (such as K3 CpG) does not form aggregates in solution and can effectively activate B cells for the production of antibodies and IL-6, but only weakly induces pDC to produce IFN. Based on the different properties of various kinds of CPGs, modifying the surface structure of CPG could solve the problem that antibodies and interferons cannot be induced in large quantities at the same time. Linking HIV TAT peptide with K-type CpG ODN to form a CpG ODN nanoring can not only enhance the adjuvant uptake but also produce IFN [8]. Moreover, Y-type, X-type and hexapod-like CPG patterns can be generated to promote the uptake of immune cells and then promote TLR9-mediated production of IFN [9].

Non-immunostimulating component antigen delivery systems which directly activate immune systems are a hot spot in current immune research. Immune response is more effectively induced by nanomaterials, because they have the size equivalent to pathogens, and they can be more easily recognized and absorbed by antigen-presenting cells [10]**.** Nanomaterials currently used for immunization mainly involve (1) polymeric nanomaterials including poly (d, l-lactide-co-glycolide) (PLG) [11] and poly(d, liter-lactic acid-hydroxy acid) (PLGA) [12–14], (2) inorganic nanostructures covering gold nanoparticles [15, 16] and carbon nanoparticles

[17, 18], (3) organic ingredients containing liposome [19, 20], autonomous protein [21, 22], and self-assembled DNA nanostructures [23–25]. The mode of antigens loaded and delivered by nanomaterials is mainly the construction of virus-like particles (VLPs), which induce a long-term production of antibodies specific to many proteins displayed on the surface of these viral particles. But when comparing with the whole cell vaccines, VLPs often show a low-level and short-lived production of antibodies [26, 27]. Therefore, many studies have focused on size control and surface modification of VLPs to enhance the VLP-mediated immune response [26]. Despite many synthetic nanoparticles have been exploited as vaccine carriers to assembly particulate antigens, DNA nanostructures stand out because they can activate both antigen-dependent signal and accessory signal to generate high-quality B-cell responses. As a result, DNA nanostructures harness the engineering potential of particulate antigens for rational design and construction of effective DNA-based vaccines by mimicking biophysical and biochemical cues from viruses [28–31].

Due to Watson-Crick base-pairing principle, the self-assembled DNA nanostructure is autonomous and programmable, and this unique feature makes it possible to utilize computer programs to design and simulate its structure and geometry [32, 33]. Furthermore, the chemical modification of DNA offers different methods to conjugate DNA to functional ligands, such as covalent cross-linking at 5′ or 3′ ends or nucleic acids base pairing [34]. DNA tile which is assembled as building block was constructed into several nanoscale devices for nanomedical applications in ligand delivery and immunization filed. While nucleic acids need transfection agents to penetrate into the cells, it has been shown that DNA nanoparticles were naturally internalized by antigen-presenting cells (APCs) in a shape- and size-dependent manner, even if they are not targeted ligands [35]. Additionally, the similarity between DNA sequences of the delivery platform and nucleic acid adjuvants, such as CpG DNA, enables DNA nanomaterials to simultaneously activate innate immunity. DNA nanomaterials currently used in immune engineering are mainly DNA tetrahedral [23], DNA hydrogel [24], DNA nanotubes [25], DNA dendrimer [36], and DNA nanoflower [37] (Fig. 7.1). These DNA nanomaterials not only share the common characteristics of nano-vaccines in terms of size and structure but also show their unique advantages. First, DNA nanomaterials are highly biocompatible. Antibodies against double-stranded DNA or DNA nanostructure are not detected in hosts after immunization [38]. It may be due to the presence of the double-stranded DNA genome in the host, which makes the host avoid the immune response against DNA that would cause autoimmune diseases [39]. Second, some DNA nanomaterials without being loaded with any immunoregulatory molecules can regulate the innate immune response by acting on immune-related signaling pathways without producing any toxic side effects [25, 40]. Third, DNA nanomaterials also have structural controllability to be used as a platform for organizing various immune adjuvants, such as CpG ODN and proteins/peptides. Various experiments in combination with immunomodulatory molecules have proven that DNA nanomaterials could (1) protect immunomodulatory molecules from degradation by enzymes and prolong the half-life in the body, (2) improve the efficiency of cells in absorbing immunomodulatory molecules, (3) deliver target

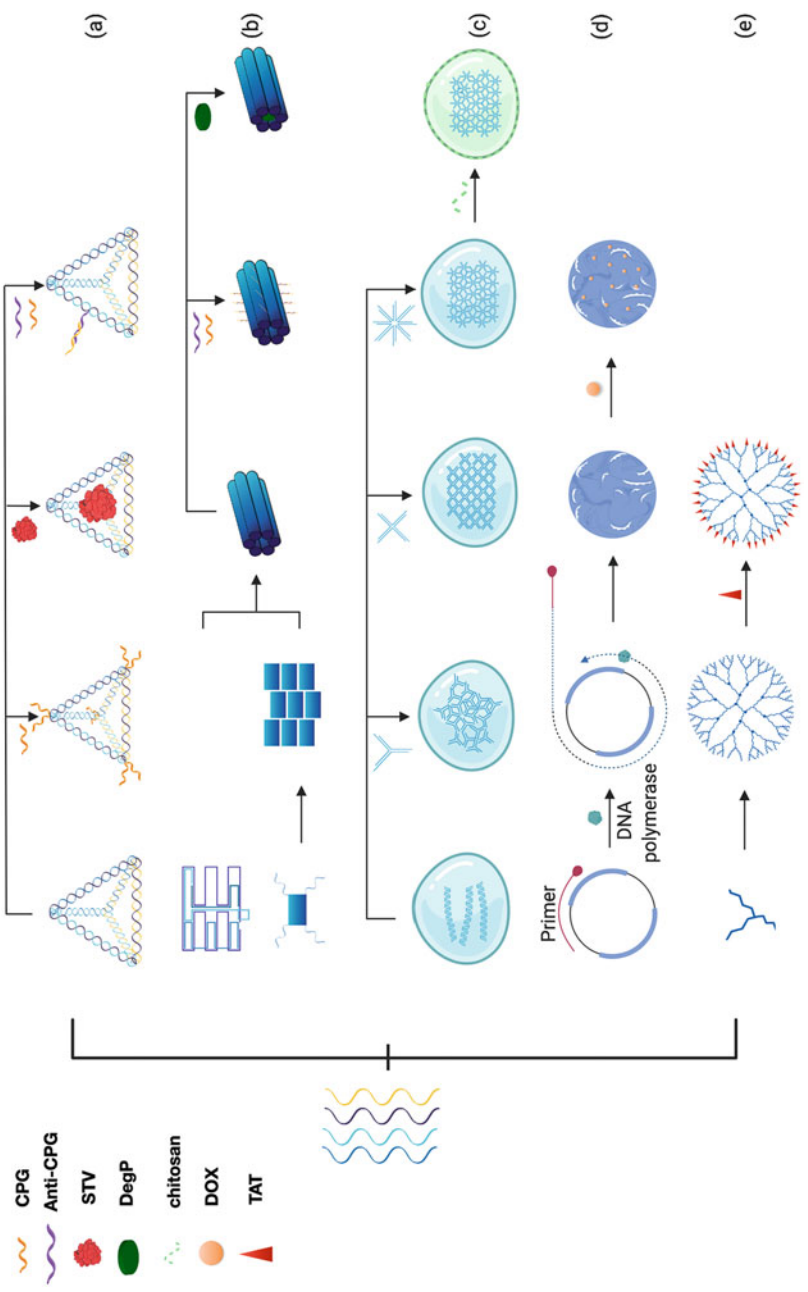

Fig. 7.1 Syntheses and structures of DNA vaccines: (**a**) DNA tetrahedron, (**b**) DNA nanotube, (**c**) DNA hydrogel, (**d**) DNA nanoflower, and (**e**) DNA dendrimer

immunomodulatory molecules to tissues, (4) change intracellular localization, and (5) change target physical form and induction of cytokines [41–44]. On the basis of the above, the performance of DNA nanomaterials can be further improved by estimating receptor/ligand interactions [38]. For example, the distance between the antigen and the agonist can be reasonably adjusted according to the controllability of its structure. When the spatial positions of the antigen and the adjuvant are close, the immunogenicity of the adjuvant is enhanced [45]. In addition, DNA nanomaterials incorporate nuclease-sensitive sequences to regulate its sensitivity to nuclease degradation, thereby reducing the host's immune resistance to the vaccine [46]. In summary, DNA nanomaterials can be used as a carrier to transport antigens and play a synergistic role with antigens to maximize the influence of vaccine adjuvant.

7.2 DNA Nanostructures

7.2.1 DNA Tetrahedron

DNA tetrahedron (TDN) is formed via self-assembly of four or more DNA single strands, based on Watson-Crick pairing principle [47]. For instance, four predesigned single strands are molar—equally added to TM buffer (Tris and $MgCl_2$) and then heated at 95 ° C for 10 min and cooled down to 4 ° C for 20 min. In one DNA tetrahedron, each single-stranded DNA forms one triangle, and three sides of the triangle are complementary with one of the other triangle. Usually, single-stranded DNA molecules with a length of 63 nt are used to construct a DNA tetrahedron with a side length of 20 bp. This DNA tetrahedron is not a solid structure but a framework nucleic acid with cavity, which could carry objects between the edges formed by the double-stranded DNA. DNA tetrahedrons are featured with excellent biosecurity and promising biocompatibility and controllable programmability. Currently, DNA tetrahedrons show promising potentials in promoting proliferation and migration of multiple types of stem cells and cell lines, such as human corneal epithelial cells [48], mouse L929 fibroblasts [49], and rat adipose-derived stem cells [50], with working concentrations below 250 nM [48], which demonstrated excellent biological safety. The biocompatibility of DNA tetrahedra is referred to its transmembrane capacity. To date, accumulating studies have found that DNA tetrahedrons can be efficiently taken up by various types of cells without any transfection agent. DNA tetrahedron was found to minimize electrostatic repulsion through corner attack mechanism and thereby quickly go across the membrane [51], depending on caveolin-mediated pathway [52]. This process usually requires the size of DNA tetrahedron to conform to initiative of cell intake. Inspiringly, after entering mammalian cells, DNA tetrahedron can remain intact for at least 48 h [53]. Based on Watson-Crick base-pairing principle, DNA tetrahedron can be modified via mainly three methods to form upgrading structural and functional transformations (Fig. 7.1a): (1) pre-linking the modifiers, like nucleic acids, at the 5 ′ or 3 ′ end of single strands before self-assembling of DNA tetrahedra;

(2) designing an overhang which would not interfere with DNA tetrahedron formation but combining with modifier through complementary sequences [54]; and (3) physical conjugating modifiers (e.g., proteins) in the DNA double helices [55]. Generally, there are two ways to dissociated the modifiers and carriers: (1) base pair mismatch, of which the degree is often related to the degree of dissociation [54], and (2) arrangement of fragile gaps between modifier and DNA tetrahedron. Such gaps are usually composed of consecutive identical bases [56, 57].

Inspired by the above three modification methods, researchers assembled CpG ODN on the DNA tetrahedron to form a complex in the field of immunoengineering. CpG ODNs are well-known immunostimulatory agents, which can be recognized by Toll-like receptor 9 (TLR9) that activates downstream pathways to induce immunostimulatory effects, secreting various pro-inflammatory cytokines including tumor necrosis factor (TNF)-R, interleukin (IL)-6, and IL-12. This TDN-CpG ODN complex can be taken up by APCs to enhance immunity. The unique properties of TDN-CpG ODN complex are, firstly, in the preparation of vaccines, the biosafety of the complex needs to be premiere considered. Li et al. tested the biocompatibility of low concentration TDN-CpG complex in cells, and the results showed that cell viability was not affected. The immune system maintains a critically organized network to defend against foreign particles. The immune system becomes active when TDN-CpG complex is applied to organisms. Many DNA nanomaterials are greatly restricted in their applications due to the potential to induce autoimmune diseases. For example, anti-double-stranded DNA (anti-dsDNA) antibodies are implicated in the pathogenesis of many autoimmune diseases. However, a study showed that after DNA tetrahedron injection for 18 days, researchers observed no detectable level of anti-dsDNA antibodies [38]. Moreover, a recent study found that tetrahedron DNA can significantly regulate the balance of NO (an inflammatory mediator) production, particularly at the dose of 250 nM. TDN can also work as a potentially useful candidates in immunomodulation to inhibit mitogen-activated protein kinase (MAPK) phosphorylation to attenuate the expression of NOIL-1β (interleukin-1β), IL-6, and TNF-α in RAW264.7 cells induced by LPS. In addition, researchers have also found that DNA tetrahedron inhibit LPS-induced reactive oxygen species (ROS) production and apoptosis by upregulating the mRNA expression of antioxidants [40]. The anti-inflammatory and anti-oxidative stress abilities of DNA tetrahedrons dispel concerns that they may cause autoimmune diseases and further proved the biosafety of DNA tetrahedron. In addition, DNA tetrahedrons are synthesized from single-stranded DNA and can be degraded and metabolized by endonuclease in organism. The metabolic products are deoxynucleotide monomers, which will not produce more toxic side effects. Secondly, the TDN-CpG ODN complex needs to be efficiently taken up by APC. Ohtsuki et al. designed and compared the uptake rate of tetrapod-like structured DNA (tetrapod DNA), tetrahedral DNA, tetragonal DNA, and single-stranded DNA (ssDNA) into macrophages [58], and results showed that DNA tetrahedron was taken up by cells nearly twice as fast as tetrapod DNA and tetragon DNA and nearly five times as fast as ssDNA, thus confirming that TDN-CpG ODN complex possesses the capacity of efficient cell uptake. Thirdly, TDN-CpG ODN complex needs to be stable for a period of time in

organism, which requires certain resistance to endonuclease. DNA tetrahedral nanostructures have been proven to be stable against nuclease degradation in biological media. The stability of TDNs has been further quantitatively analyzed by incubating the same concentration of TDN and double-stranded DNA in 50% non-inactivated fetal bovine serum [23]. Weakened TDN band could still be observed after 24 h, while the DNA double-strand was completely degraded after only 2 h. Additionally, co-localization study using dual-labeled nanostructures (Cy3 and Cy5 labeled on different vertexes) showed that the two fluorescent colors were present nearly in the same place even after 8 h, which further confirms that DNA nanostructures are intracellular stable. Compared with other CpG carriers, such as liposomes, the free arrangement of the four bases provides a high degree of freedom for DNA tetrahedrons and can be programmed to design sequences that meet different needs. Using the programmability of DNA tetrahedrons, Zhang et al. incorporated a biotin moiety at the 5′ end of DNA single-strand and self-assemble the DNA upward. Each surface of the DNA polyhedra displays three biotin moieties, related by a threefold rotational symmetry [59]. Overall, the excellent properties of the CpG-TDN complex suggest its potential for application in immunoengineering. To further assess its ability of stimulating immunity, Ohtsuki et al. incubated 6 μg/mL CpG-TDN and CpG ODN with human PBMCs; as a result, cell treated with CpG-TDN for 24 h expressed twice the amount of IFN-α by comparison to the CpG-ODN-treated cells [58]. But after adding chloroquine, an inhibitor of endosomal TLR signaling and IFN-α releasing from human PBMCs were strongly inhibited, highly suggesting that the IFN-α release after addition of CpG-TDN complex occurred through TLR9 pathway. This result indicated that loading on TDN directly or indirectly enhanced the immunostimulatory capacity of CpG ODN. To investigate the impact of different concentrations of CpG-TDN, two sets of varying CpG-TDN concentrations (2 μg/mL and 6 μg/mL) were constructed by incubating with RAW264.7 cells for 8 h, and it has been found that CpG-TDN concentration was positively correlated with TNF-α expression [58]. Another study showed that the expression of TNF-α induced by the CpG-TDN complex was more than five times higher than that of the CpG carried by Lipofectin. In addition to TNF-α, other cytokines also play a role in CpG-TDN-mediated immune activation. After adding CpG-TDN complex to RAW264.7 cells for 8 h, high levels of IL-6 and IL-12 expression were also detected. The results of ELISA assays showed that the expression level of IL-6 can reach more than 60 pg/mL and the expression of IL-12 can reach more than 200 pg/mL [23]. TNF-α, IL-6 and IL-12 were all secreted by the activation of the TLR9 pathway, which suggested that the CpG-TDN complex can produce a stronger immunostimulatory capacity through the TLR9 pathway than the CpG ODN, but whether there are any other signaling pathways or cytokines involved in the immune-activation process remains to be elucidated. Cellular uptake efficiency and stability significantly enhances the immunostimulatory capacity of the CpG-TDN complex, but this cannot be taken as strong evidence for its significant difference from Lipofectin. Some studies elucidated the mechanism of CpG-ODN's powerful immune-stimulating ability from multiple perspectives. Exposure of the 5′ end of CpG ODN is closely related to its immunostimulatory activity. Conjugation at

the 5′ end will significantly inhibit the immunostimulatory activity of CpG DNA, while the conjugation at the 3′ end won't, and this difference does not owe to the difference in cell uptake capacity, which indicates that the receptor reads the DNA sequence from the 5′ end [60, 61]. Further research showed that CpG ODN was often composed of stimulatory and structural domains. Different combinations of stimulatory and structural domains can stimulate the immune activation of different cell lines, suggesting that the secondary structure formed by the CpG-TDN complex may be one of the reason for the strong immune stimulation ability [62, 63]. In addition, the physical aggregation state of CpG ODN is also related to its immunostimulatory ability [64]. Studies showed that CpG aggregates can induce bone marrow-derived monocytes to secrete more IL-12 than CpG ODN, indicating that it has stronger TLR9 binding ability [65]. Recently, attention had been paid to the relationship between the number of CpG motifs and the immune activity of CpG-TDN. Li et al. found as the number of CpG motifs increased the immune stimulatory effect was enhanced [23]. The enhancement was not only due to the increased concentration of CpG which leads to an increase in the affinity of TLR9 but also due to the common effect of the four CpG motifs caused by the spatial structure of DNA. Because the DNA tetrahedron has a uniform size and precise structure, the CpG motif can be accurately placed at any specific position of the tetrahedron for predetermined sequence number and sequence design. The accurate correspondence is beneficial to the recognition between the CpG sequence and TLR9. Based on this, the efficacy of DNA nanostructures can be further improved.

In order to further explore the application of DNA tetrahedrons in the field of immunity, researchers used the structural properties of DNA tetrahedrons. Since these cage-like nanostructures are hollow structures, they are able to assemble with subunit proteins into virus-like particles (VLPs) [66]. VLP represents a major breakthrough in vaccine development. It is considered to better induce immune response. Previous studies have shown that the size, shape, surface charge, hydrophobicity, hydrophilicity, and receptor interactions of an antigen can affect APC's absorption [26]. Although direct connection of CpG ODN with antigen has been shown to induce a strong B-cell response [45], it is not feasible to use it to prepare vaccine directly. Therefore, effective carriers carrying CpG ODN and antigen are required to prepare more complex and useful vaccine. Recombinant DNA technology assembles subunit proteins into VLPs [66, 67], which is similar to natural virus structures, but without viral genetic material. The immunogenic epitopes displayed on VLPs can induce a strong immune response. Therefore, VLPs were widely studied as an effective and safe platform for assembling target epitopes against many pathogens and tumors [68]. At present, DNA tetrahedrons are used to construct VLPs. CpG ODN is connected to the vertices of tetrahedrons, and antigens are connected to each face of the tetrahedron through biotin. By increasing the number of biotins, this connection can be strengthened, which can solve the great challenge for DNA-directed guest organization [69–71]. TDN-VLP is constructed by three steps: (1) conjugation of CpG ODN and biotin moiety at the end of DNA single strand, (2) the programmed self-assembly of DNA tetrahedron, and (3) immobilization of proteins onto the DNA scaffolds [59]. The currently reported TDN-VLP only

carries streptavidin (STV) as antibody and CpG ODN as adjuvant. But the successful deployment of STV also highlights that DNA tetrahedron scaffold has the potential to organize a wider range of objects, which can be applied to develop other VLP vaccines by mounting other antibodies as needed. To verify the immunostimulatory ability of TDN-VLP, particularly in elicit an antibody response against the model antigen, BALB/c mice were treated with experimental artificial immunization protocol including three steps: (1) primary immunization, (2) secondary immunization, and (3) antigen challenge. The time intervals between primary and secondary immunization and antigen challenge were 28 days and 24 days. Compared to those immunized with free CpG + STV and STV only, the TDN-VLP complexes induced a stronger and longer lasting anti-STV antibody response, partially due to the generation of STV-specific memory B cells. Quantitative analysis of anti-STV IgG antibodies expression level was processed by ELISA, and results showed that TDN-VLP induced twice the antibody secretion of free STV + CpG, even after 60 days of antigen challenge. To directly evaluate the long-term immunity induced by various immunization regimes, researchers applied an enzyme-linked immunosorbent spot (ELISPOT), assay resulted that significantly elevated levels of specific antibody-secreting cells (ASCs) were found in mice immunized with the TDN-VLP complex compared to those immunized with free CpG + STV and STV only, and ASCs were transformed from memory B cells after STV stimulation in vitro, which indirectly proved that TDN-VLP can induce the generation of memory B cells. The CpG-TDN complex can only elicit a short-acting immune response because it mainly acts on T cells and only induces upregulation of multiple cytokines, but does not promote the generation of memory B cells. TDN-VLP can induce the generation of memory B cells and establish long-term and efficient artificial immunity, which is the goal pursued by vaccination. The reason for such a significant difference may be that the TDN-VLP complex better mimics the natural virus structure. Through the programming of TDN, the spatial arrangement of each immunogenic component can be controlled to meet the needs of receptor recognition. However, the recognition receptors and downstream signaling pathways that induce long-term immunity by TDN-VLP still need to be further studied. The influence of the spatial arrangement of various components on immunogenicity has not yet been elucidated, which is of great significance for the rational design of VLP.

Overall, DNA tetrahedron as a carrier, its size, charge, and other physical properties meet the requirements of internalization by APC. Base pairing can also allow DNA tetrahedra to inherently carry CpG motifs, which has unique advantages over other vaccine vectors. Because the close proximity of antigens and adjuvants is essential to enhance the immunogenicity of vaccines, programmable DNA tetrahedrons provide multivalent and three-dimensional configurations. Therefore, DNA tetrahedrons can be considered as an excellent platform for constructing vaccines that mimic virus-like particles. Additionally, the 3D spatial arrangement of each immunogenic component can be easily controlled through the rational design of the tetrahedral sequence, so that the DNA tetrahedrons can meet the spatial structure requirements for inducing the optimal immune response. Most importantly, DNA

tetrahedrons have better safety because they can regulate the oxidative stress and inflammatory response of macrophages and can effectively prevent the occurrence of autoimmune diseases. The above characteristics complement each other, making DNA tetrahedron a potential vaccine preparation platform in the field of immune engineering.

7.2.2 DNA Nanotubes

Among various artificially synthesized nanotubes, biomimetic DNA nanotubes have attracted widespread attention due to their design flexibility. Two methods can be used to prepare structurally stable DNA nanotubes. One is programmable assembly of DNA magnetic tiles [72] (Fig. 7.1b). The DNA tile consists of a DX molecular core and four single-stranded sticky ends which allow it to bind to other tiles. Given an appropriate set of sticky ends, DNA tiles will form a lattice sheet by adjusting the curvature of the phosphate skeleton and the location of the sticky ends. After assembly, DNA tiles form an angle with each other, and the flat sheet becomes tubular. The other method is to plicate layers of double helices to a honeycomb lattice [73]. With the help of caDNAno software, honeycomb DNA origami tubes can be easily designed [32]. As one of the candidate carriers of nano-vaccine, DNA nanotubes have excellent stability, flexible loading capacity, and remarkable biocompatibility. The robustness of Watson-Crick base pairing ensures a programmable and sophisticated design of various types of DNA nanotubes. DNA nanotechnology allows bottom-up assembly of complicated nanotube structures ranging from a few nanometers to micrometers in size, able to load functional nucleic acids, proteins, peptides, and organic and inorganic materials. Additionally, DNA nanotubes also show promising biological properties. Upon exposure to multiple endonucleases [33], including DNase I, T7 endonuclease I, T7 exonuclease, *Escherichia coli* exonuclease I, lambda exonuclease, Mse I restriction endonuclease, and lysates from various cell lines [74], DNA nanotube can still maintain its structural integrity for 12 h. Furthermore, a higher cell-permeable efficiency of DNA nanotubes with greater rigidity was observed compared to that of spherical, circular, or other DNA nanostructures [75, 76]. Due to the larger contact area with cell surface and cross-linking membrane receptors, CpG-modified DNA nanotubes are more easily to be internalized than single spherical DNA-adjuvant complexes or single-stranded CpG motifs. Recent study suggests that the efficient internalization of cells is also due to the corner attack mechanism which indicated that the cell entry of DNA nanostructures in the range of several tens of nanometers is not related to their size but to the shape, and the anisotropic structures are more likely to enter cells than isotropic structures [51]. Overall, these characteristics make DNA nanotube an efficient vehicle for the delivery of CpG.

Currently, methods to modify CpG onto DNA nanotubes are (1) adding single-stranded DNA handles that protrude from the wall of the DNA origami tube to the defined position, meanwhile combining anchor sequences which complementary to

the handles with CpG, and CpG is connected to the nanotube by base pairing. (2) Wrapping modifiers (e.g., proteins) in the hollow structure. Studies showed these CpG-modified DNA nanotube complex could trigger immune responses. Mammadov R et al. [77] conducted nanotubes with a diameter of 10–15 nm and a length >200 nm, via using CpG ODN and β-sheet-forming peptides. Compared to spherical nanostructures and CpG ODN, the nanotubular structures induced higher levels of IFN-γ expression and lower levels of IL-6 expression. More importantly, the nanotubular structure can also synergize with CpG ODN itself and induce higher levels of CD86 expression, which proves that the immune response to Th1 phenotype induced by CpG-DNA nanotube is more effective in defending against intracellular pathogens. The role of the nanotubular structure and the CpG ODN is not superimposed on each other but rather a synergistic effect of mutual promotion. Under this effect, using the nanotubular structure of the CpG ODN will improve the adaptive immune response to the vaccine complex by allowing more CpG ODN loaded and spatial synergies. Currently, researchers have developed a hollow tube-shaped DNA origami structure consisting of 30 parallel double helices with maximized surface area for both 62 inner or 62 outer binding sites for CpG anchor sequences (CpG-H0s) [25]. These nanotubes can be efficiently internalized by antigen-presenting cells, while protecting CpG sequences from degradation and inducing high local concentration of CpG in vivo, suggesting a high-intensity immune response. As entering antigen-presenting cells, CpGs dissociated from carrier tubes and bound to TLR9 receptor of endosomal membrane. Compared with the equivalent amount of free CpG-H0s, CpG-H0-modified DNA nanotubes triggered a higher cytokines secretion with more than fivefold of CD69 expression by dendritic cells. Compared to Lipofectamine, a commonly used lipid transfection reagent, DNA nanotubes can induce higher levels of IL-6 and CD69 expression but lower cell viability. Interestingly, DNA nanotube itself was reported with the ability to activate innate immunity through a non-TLR9-mediated pathway. However, if the CpG sequence is decorated in the DNA tube, immune stimulation is mainly performed through the TLR9-mediated pathway. These traits should be considered when DNA nanotubes are used in future vaccine vectors.

Besides inducing immuno-related cytokine expression, DNA nanotubes can also induce the recruitment of leukocytes. Forty-eight different oligonucleotides are temperature-controlling assembled into eight parallel double helices to form a DNA nanotube, combined with 20 nt CpG ODN [78, 79]. This complex is found stable in serum at different normal tissue-like concentrations and can significantly increase TNF-α expression levels in RAW 264.7 macrophages. In vivo study suggested that NF-κB pathway and TLR9-mediated immune response were involved. Within 5 min after venous microinjection in the cremaster muscle, DNA nanotubes were rapidly internalized by resident cells attached to blood vessels and tissues around the injection site. Inspiringly, a significant recruitment of leukocytes into the target tissues, depending on the activation of mast cells, was also observed. Mast cells were close to the inner side of capillary cavity, quickly degranulated after receiving cytokines secreted by macrophages [79, 80], then released pro-inflammatory mediators [81, 82], and increased leukocyte' stickiness [83–85],

allowing leukocytes to be expelled from the venules behind capillaries. This phenomenon could not be caused by ordinary DNA nanotubes or CpG ODN, indicating DNA nanotube as a potential vehicle for targeted macrophage recruitment, but its mechanism is not clear. For a long time, the low affinity of proteins to DNA nanotubes limits its application in the field of vaccine. Recently, Sprengel et al. wrapped DegP protein in an envelope-like hexagonal DNA prism, with weak non-covalent interactions on protein surface, which protected its natural state [86]. Such DNA nanotubes are theoretically suitable for any type of protein recognition motif and are able to overcome the low affinity for ligand binding. It is expected that this structure can be used to encapsulate specific antigens and adjuvants after being modified in the lumen and plays a role in the assembly of vaccine.

In summary, with high biocompatibility, accurate design of the nanoscale cavity, and multiple ordered modification sites to facilitate the deployment of immunostimulants and the ability to recruit leukocyte, DNA nanotubes have opened up broad prospects in the field of immune engineering.

7.2.3 DNA Hydrogel

DNA hydrogels are formed by cross-linking different DNA monomers into a 3D network [87] (Fig. 7.1c). By changing the type and concentration of DNA monomers, DNA hydrogels have been designed to enable a variety of biomedical applications, including drug delivery, cell encapsulation, and immune regulation [88, 89]. Sequence-based immunostimulatory and immunosuppressive effects have been identified in DNA hydrogels [88, 90]. Compared to its DNA strand components, cross-linked DNA gels are more physically and chemically stable which often take longer to be degrade. Extending the retention time of DNA hydrogels in the body may help to enhance immune response and develop adaptive immunity in cancer treatment [88].

The DNA hydrogel structure which consists of Y scaffolds with three CPG ODN single chains and linkers were confirmed to be rapidly formed without any chemical treatment and can thermally stimulate by switching between gel and sol states within the transition temperature. Therefore, the local temperature changes between normal tissues and tumor areas have shown huge potential in the concentration and induction of immune responses at tumor sites and exert antitumor effects [91]. DNA supramolecular hydrogel vaccine (DSHV) which was formed from Y-type DNA hydrogel with P1 antigen was applied to the top of macrophage RAW264.7 cells which stained with CM-Dil dye at 37 ° C for 30 min. DSHV system inherited the self-healing properties of DNA supramolecular hydrogels which can ensure sufficient mechanical support for close contact between cells and immunostimulants/antigens, which was able to induce a strong immune response. The migration of RAW264.7 cells was observed that the cells migrated up 100 μm into the DSHV about 1 h with a turntable confocal laser scanning microscope. This antigravity movement of the cells proved that DSHV can effectively recruit macrophages. At the

same time, after the cells passed through the DSHV, no obvious channels were left. Detecting with ELISA reagent, it was found that the DSHV group could strongly produce 365 pg/mL IL-6 and IL-12 pg/mL IL-1, which exerted the most influence on cytokine-inducing effect compared to the control group [92]. Two types of X-DNA were constructed using four oligodeoxynucleotides; one contains six valid CpG motifs (CpG X-DNA) and the other not (CpG-free X-DNA). CpG X-DNA hydrogel was more effective than its components and the hydrogel without CpG on the production of TNF-α from mouse macrophage-like RAW264.7 cells and the maturation of mouse dendritic DC2.4 cells. The cytotoxic effects of X-DNA, doxorubicin (DOX), and their complexes (DOX/X-DNA) were examined in colon26/Luc cells which are murine adenocarcinoma clones stably expressing firefly luciferase and RAW264.7 cells co-culture systems. Among of them, DOX/CpG X-DNA showed the highest ability to inhibit colon 26/Luc cells proliferation and colon26/Luc subcutaneous tumor growth by slowly releasing DOX from CpG DNA hydrogel. These results indicated that CpG DNA hydrogel was an effective continuous system that transmits CpG DNA to TLR9 positive immune cells and DOX to cancer cells [24]. Hexapod-like DNA (hexapodna) hydrogels were composed of six ODNs with unmethylated CpG sequences. An in vivo study showed DNA hydrogels were more resistant to degradation than hexapodna in DNase buffer solution and had a higher level to induce IL-6 released by cells than hexapodna and CpG-ssDNA. IL-6 expression was present at the site where the hexapod or DNA hydrogel was injected for 6 h. After 24 h, the IL-6 expression remained high only in DNA hydrogels and was observed in draining lymph nodes. However, after injecting DNA hydrogels into the skin, the IL-6 concentration in serum did not increase significantly, indicating that the DNA hydrogels only induced IL-6 expression in the local location where the hydrogel aggregated. When loading ovalbumin (OVA) with DNA hydrogels, the OVA/DNA hydrogels were found significantly increased the content of OVA-specific IgG in mouse serum and stimulated spleen cells to produce higher amounts of IFN-γ. Besides, OVA/DNA hydrogels could induce cytotoxic T-lymphocyte (CTL) response against EG7-OVA tumors in mice. Compared to complete Freund's adjuvant (CFA) and alum-injected OVA used in some vaccine formulations, OVA/DNA hydrogels did not cause significant changes in the injection site or spleen weight. However, the formation of the hydrogel delayed the clearance of CpG DNA and OVA to increase the activity of CpG DNA immunostimulatory and enhance the immune response of OVA, which indicates that the OVA/DNA hydrogel can act as an antigen, and did not cause obvious harm in vivo [88]. Chitosan is a biocompatible cationic polymer that can electrostatically interact with DNAs, which is further studied by mixing OVA/hexapod-like DNA hydrogels and chitosan (chitosan-OVA/DNA hydrogel) and injecting into mice. Compared with simple sDNA hydrogel, the structure of chitosan-OVA/DNA hydrogel was more stable and tougher, which lead to OVA antigen released more slowly and remained longer in the injection site. Compared with the OVA/DNA hydrogel, the chitosan-OVA/DNA hydrogel had higher level of serum OVA-specific IgG induction by intradermal immunity. These results indicated that chitosan-OVA/

DNA hydrogel was an improved sustained release preparation for effectively inducing an antigen-specific immune response [93].

DNA hydrogel not only plays a role in the stimulation of immune response but also suppresses immune responses for the treatment of autoimmune diseases. It has reported that activation of TLR9 can exacerbate autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [94]. Therefore, TLR9 inhibitors have great potential as therapeutic agents for such inflammatory diseases. TLR9 antagonistic ODNs which called immunosuppressed oligodeoxynucleotides (INH-ODNs) combined to a structure similar to the Chinese character Takumi and then processed it into a higher-order hydrogel. Flow cytometry analysis and confocal microscopy revealed that TNF-α and IL-6 activity were reduced in mouse macrophage-like RAW264.7 cells and DC2.4 dendritic cells. Compared to iTakumi and iODN1 which is a sense of iTakumi, iTakumiGel more effectively inhibited the release of TNF-α, and iTakumiGel showed the highest inhibitory effect, which may relate to the decrease of CpG uptake by immune cells [90]. The more complex the structure of the nucleotide molecule is, the greater the absorbing efficiency of immune cells have when the total number of nucleotides is the same [95], so the complex structure of the iTakumiGel promote cells to uptake INH-ODNs. This result indicated that Takumi-based DNA hydrogels could be used to deliver INH-ODNs to macrophages and dendritic cells to inhibit TLR9-mediated over-induction of pro-inflammatory cytokines, which showed its potential for treating autoimmune diseases [90].

In summary, different types of DNA monomers have diverse qualities, which result in two capabilities of DNA hydrogels that enhancing the innate immunity and adaptive immunity by prolonging the action time in the body and inhibiting the immune response to treat autoimmune diseases.

7.2.4 DNA Nanoflower

DNA nanoflower (NF) is formed with two types of DNA strands (a template and a primer) and replicates through rolling cycles (RCR) which is an isothermal enzymatic reaction involving many circular genomic DNAs (such as plasmids or viral genomes) to generate long components (Fig. 7.1d). Without relying on Watson-Crick base pairing, NFs are not self-assembled using conventional short DNA, but long structural units are obtained through liquid crystal synthesis, which helps NFs maintain high stability. The main reasons of their stability are the following: (1) long structural units avoid other nicking sites being sensitive to nuclease cleavage; (2) extensive inter-strand and intra-strand weaving of stable DNA building blocks to prevent denaturation or dissociation; (3) each NF is equipped with high density DNA, thereby reducing the probability of nucleases access to NF; and (4) even if the outer layer of NF is dissociated, its inner layer can maintain its function [96]. Because of its assemblability and biosecurity, it is widely used in drug loading, transportation [97], and biological imaging [98]. In order to optimally deliver CpG ODN to

stimulate the immune cell response, CpG ODN must be internalized into the cells, especially to the endosome. Due to suitable size of NF is from 100 to 300 nm, CpG NF which consist of CpG ODNs is easily captured by macrophages and activate the immune system [99].

DNA nanoflowers can excrete TNF-α and IL-6 by TLR9 immune pathway. After incubating macrophages with 100 and 20 nM CpG-NF, free CpG, and free CpG-liposome for 8 h, ELISA analysis showed that CpG NF induced TNF-α and IL-6 secretion level was significantly higher than that induced by free CpG or CpG-liposome. Even when the concentration of CpG NFs was reduced to 10 nM, their induction still caused the saturation level of TNF-α secretion. Additionally, NFs specifically stimulated the proliferation of immune cells when they were incubated with RAW264.7 cells for 24 h [100]. Moreover, NFs can trigger the proliferation of macrophage-like cells through its immune stimulation, thereby stimulating the secretion of immune-stimulating cytokines that induce apoptosis and necrosis of cancer cells [101]. The efficacy of NFs has been proved by analyzing the co-culture supernatant of CCRF-CEM cells (T-lymphocyte leukemia, suspension cells) and RAW264.7 macrophages with flow cytometry. The result showed that the percentage of CCRF-CEM cells treated with CpG NF was significantly reduced compared with the control NF or free CpG treated groups. In addition, the inhibitory effect increased with extension of treatment time [100]. Cancer chemotherapy is partially hindered by side effects and multidrug resistance (MDR), which are partly caused by drug efflux of cancer cells [102], so that it is urgent to require a targeted drug delivery system to circumvent MDR. NF loading with Dox is a potential platform for circumventing drug resistance during targeted anticancer drug delivery. It has PH adaption capability which is stable at physiological pH and promotes drug release under acidic or alkaline conditions. An experiment transported NFs with leukemia cell aptamers KK1B10 (KK) to deliver Dox showed that the same concentration of KK-NF-Dox was more stable and the release of Dox from NF-Dox was slower under the condition of PH 7.4 compared with the rapid release of free Dox. At pH 5 and pH 9, the release of Dox was greatly accelerated, and its release rate was about half of the free Dox diffusion rate. Therefore, NF-Dox can transport Dox steadily during drug delivery and promote the release of Dox when accessing to acidic subcellular organelles such as endosomes and lysosomes. In short, DNA NFs can prevent drug outflow and strengthen the retention of drugs in MDR cells, thereby avoiding MDR and reducing side effects [37].

Intertwining DNA-RNA nanocapsules (iDR-NC) is consist of DNA CpG and STAT3 short hairpin RNA (shRNA) by using micro-flower nano-systems, subsequently shrunk by PEG-grafted polypeptide (PPT-g-PEG) copolymers. The nanocapsules act as a vaccine carrier based on following characteristics: (1) NC improves the delivery efficiency of lymph node at the tissue level and APC at the cell level; (2) acid-labile PPT not only ensures solubility of the high-level copolymer and effective MF contraction rate but also promotes intracellular delivery by enhancing the proton sponge effect after PEG shedding to expose cationic PPTs in acidic endolysosomes; and (3) hydrophobic PPT allows tumor-specific neoantigens to be loaded into iDR-NC through the hydrophobic interaction between peptide

neoantigens and PPT to co-deliver adjuvants and antigens [103]. Janus kinase/signal transduction and transcription activation (JAK/STAT) pathway has been targets of cancer immunotherapy [104], which can inhibit APC by various mechanisms, such as induction of antigen-specific T-cell tolerance immune response and suppression of CPG-activated immune response [105]. Therefore, it is necessary to activate TLR9 pathway and inhibit STAT3 pathway for clinical cancer immunotherapy [106]. Because of its special property, it usually acts as a vaccine carrier for vaccine delivery. An animal study showed that 18.2% and 25.4% iDR-NCs were effectively delivered to DCs and macrophages, after subcutaneously co-delivering iDR-NC and CSIINFEKL which was an epitope of chicken OVA with a cysteine appended on the N-terminal. In addition, CD80 expression in DCs and macrophages also increased, which indicate APC is activated after iDR-NCs injection. When injecting iDR-NC combined with Adpgk which was a neoantigen generated by MC38 tumor mutations into C57BL/6 mice, the results showed that the compound would elicit a strong and durable antitumor T-cell response. Besides, the compound also exert a negative impact on tumor growth. Compared with the free Adpgk, the mice treated with iDR-NC/Adpgk have five times lighter lung tumor, and the radioactivity of lung and tumor marker ^{18}F-fludeoxyglucose (FDG) was also significantly lower. Therefore, iDR-NC/Adpgk compound triggers strong and durable tumor-specific antitumor immunity [103].

In conclusion, due to the PH adaptability of DNA nanoflower, it can play a powerful role in drug transport. And after modifying and assembling it, DNA nanoflower can not only activate the immune system but also exert a strong specific antitumor effect.

7.2.5 DNA Dendrimer

Dendrimer is a well-defined synthetic spherical polymer; it is composed of Y-type DNA building blocks (Y-DNA). Y-DNA consists of a rigid arm and a specially designed hybrid region that becomes a sticky end, based on which DNA dendrimer (DL-DNA) is synthesized by a controlled enzymatic ligation method and becomes a highly charged and void-containing macromolecular tree-like architecture (Fig. 7.1e). DL-DNA has a series of interesting chemical and biological properties. The chemical properties include multiple surface functional group ends on its surface, which can be used to couple biological related molecules, and the surface groups can also be precisely heterofunctionalized by programming [107]. Due to the anisotropy and biodegradability of DL-DNA, antigens can be combined with it in various ways, thereby overcoming the problems of low cellular absorption efficiency, insufficient release of intracellular antigen, and low efficiency of antigen targeting in antigen delivery. In addition, the vector has the property of transferring nucleic acid into cells without any other transfection reagents, giving it the potential to target and deliver nucleic acid of pathogen by forming a virus-nonviral hybrid system. It can further adapt to specific cells by binding specific ligands. On account

of its programmability and great flexibility, the system can realize the targeted delivery of antigen components, which may set a promising platform for DNA vaccines [108].

Y-shaped oligodeoxynucleotides (Y-ODNs) were prepared using three ODNs with the halves of each ODN being partially complementary to a half of the other two ODNs. Y-ODN induced greater expression level of TNF-α and IL-6 from RAW264.7 macrophage-like cells than conventional single-stranded ODN (ssODN) or double-stranded ODN (dsODN); therefore, Y-type CpG DNA was more immunostimulating than the other CPG motifs [109]. Subsequently, DL-DNA was prepared by linking Y-DNA monomers and had 12 or 24 efficient CpG motifs in a unit. In order to determine the difference of immunostimulatory between Y-DNA mixture and DL-DNA, researchers mixed Y0-DNA and Y1-DNA at a molar ratio of 1:3 to generate DL-DNA (G1), then connect 6 Y2s at the end of G1 to generate DL-DNA (G2), and generate DL-DNA (G3) in the same way. G1, G2, and G3 were compared under conditions that did not include/include the immunostimulatory CPG motifs. Under non-CPG pattern conditions which contained 24 CG dinucleotide sequences but no potent immunostimulatory CpG motifs, it was found that the addition of DL-DNA (G2 and G3) induced RAW264.7 cells to secret TNF-α 2 to 50 times as much as Y-DNA mixture. And compared to the Y-DNA mixture, DL-DNA induced the cells to secrete IL-6 which is about three to five times. In addition, under the conditions of concentrations of 6 μg/mL and 18 μg/mL, the amount of TNF-α secreted by cells with a larger molecular weight G3 was about 1.3 or two times higher than that with a smaller molecular weight G2. These results indicated that DL-DNA itself had stronger immunostimulatory activity than Y-DNA. Further study also found that the molecular weight of DL-DNA was positively correlated with its immunostimulatory ability in a certain range. The molecular weight determines the size of the dendritic structure, and the particle size of G3 DL-DNA is about 20–36 nm, which is within the optimal radius of spherical granule cells to be absorbed within 27–30 nm, so macrophages can enhance the uptake of G3 DL-DNA. In another group containing CPG motifs, the addition of CpG ssDNA or CpG dsDNA induced a little secretion of TNF-a in RAW264.7 cells, but high concentration of G3 DL-DNA (18 μg/mL) can significantly enhance the secretion of TNF-α and IL-6 by about 100 times in a high concentration-dependent manner compared with Y-DNA. It was indicated that DNA immunostimulatory activity containing CpG motifs could be significantly enhanced by the formation of dendrimer-like structures. After the DL-DNA is taken up by the cell, the mechanism that can induce the cell to release a large amount of cytokines may be as follows: (1) its large branched structure leads to the reduction of active site that the nuclease can contact, thereby slowing the DNA in the cell of degradation. (2) Its unique branch structure increases the chance of being recognized by TLR9. (3) It has more CPG ODN 5′ ends for receptor recognition and subsequent immunostimulation. Therefore, due to the unique advantages of DNA dendritic structure, it can not only enhance the uptake of immune cells but also further induce immune cells to secrete cytokines to maximize the immune response [110]. Besides Y-DNA, researchers also used other monomers to construct the DNA dendrimer.

DNA strands with different combinations of hexapod, tetrapod, and tripod were designed as dendritic nanomaterials to immunize macrophages showed that under the combination of hexapod and tripod, the nanomaterial could be taken up by RAW264.7 cells and induce cytokine liberate TNF-α maximally. For hexapod-tripod dendritic structure, the more number of branches, the less expression of TNF-α by macrophages. Because the immune response induced by hexapod-hexapod dendritic structure was less than that induced by hexapod-tripod dendritic structure, it constitutes an opposite point of view to the previous conclusion that the more branches of the polypods, the stronger immune response was induced [9]. And in terms of the uptake mode, the dot-like distribution of fluorescent signals in the cells indicated that RAW264.7 cells had the same mechanism for taking in dendrimers and polypods. According to the molecular size of the hexapod-tripod structure which was the largest of all structures, this experiment speculated that the ability of nanomaterials to induce immune responses in cells may be related to the molecular size of nanomaterials, indicating that larger DNA assemblies can be more effectively absorbed by cells than smaller DNA assemblies [36].

Dendritic DNA can also interact with other molecules to induce immune responses. TAT peptide is a cell penetrating peptide and can target the endosomes of macrophages. It can be linked to DNA dendrimers to enhance cell membrane permeability and increase the accumulation of nanocarriers in the intracellular and endosomes of macrophages. Loop-CpG consists of a single-stranded loop composed of 30 nucleotides containing three unmethylated CpG motifs, an 11 bp double-stranded stem, and a sticky consisting of 12 nucleotides 5′- end [36], which can induce more TNF-α and IL-6 than Y-CPG alone. In order to combine the advantages of the two, a macromolecular polymer containing TAT and loop was constructed and evaluated. Mixing TAT-DNA conjugate with loop-CpG at 16: 1 to form CPG-loop-TAT to stimulate the immune response of macrophages. The result showed that G2-loop-TAT could induce macrophages to produce more TNF-α and IL-6 cytokines through the TLR9 recognition pathway compared to TAT and G2-loop control groups [111]. The reason why CPG-loop-TAT has stronger immunostimulatory activity may be (1) the hairpin and dumbbell structure of DNA is more resistant to endonuclease degradation than single-stranded DNA [112]. (2) CpG loop DNA on dendrimers can enhance the stability of CpG ODN in the biological environment by blocking the open end of CpG ODN, thus further stimulate the uptake of cells [111]. (3) Dendrimer nanostructures is about 33.6 to 46.6 nm, which will promote the absorption of CpG-loop-TAT by cells [113]. (4) There are 48 CpG motifs in the DNA dendrimer, each ring structure has three adjacent CpG sequences, and the structure of a unit of multiple CpG promotes the interaction with TLR9, thereby enhance the immune response [114].

In summary, DNA dendrimers have the great properties to meet the demands of effective immunostimulatory compounds (adjuvants) and improve the efficiency of vaccines, so that dendrimers can provide molecularly defined multivalent scaffolds to produce highly defined conjugates of small molecule immunostimulants and antigens.

7.3 Challenge and Prospect

DNA nanomaterials have splendid assemblability and immunity, so they can be used as ideal vaccine adjuvants in clinical applications. The assembled DNA vaccine particles can not only promote antigen formation but also deliver and retain antigens in secondary lymphoid tissues. When co-delivering with antigen and adjuvant to antigen-presenting cells, the components are able to stimulate adaptive immune response [38] (Table 7.1). Therefore, this article mainly introduces DNA tetrahedra, DNA hydrogel, DNA nanotubes, DNA dendrimer, and DNA nanoflower to explain the application of DNA nanomaterials in the field of vaccines.

To date, the main challenges faced by DNA nanostructures are the following: (1) DNA nanostructures are structurally unstable in a physiological environment and

Table 7.1 Various types of DNA vaccine

DNA structure			Immunoreaction	References
DNA tetrahedron	DNA ODN	Connect to CPG	TNF-α↑	[61]
	DNA ODN	Connect to CPG	Activate TLR9 pathway	[23]
	DNA ODN	Connect to CPG and STV	High-level antibody production, memory B-cell production	[38]
	DNA ODN	Itself	Inhibition of MAPK pathway	[40]
DNA nanotube	CPG ODN	Itself	IFN-γ↑	[79]
	DNA ODN	Connect to CPG	Activate TLR9 pathway	[25]
	DNA ODN	Itself	Activate non-TLR9-mediated pathway	[25]
DNA hydrogel	Y-CPG	Connect to P1	Recruitment of macrophages; IL-6↑; IL-12↑	[92]
	X-CPG	Connect to DOX	TNF-α↑; Inhibit the growth of adenocarcinoma cells	[24]
	Hexapod-CPG	Connect to OVA	IL-6↑; IgG↑; Induce CTL response	[88]
	Hexapod-CPG	Connect to OVA and chitosan	IL-6↑; IgG↑↑; Induce CTL response	[93]
	iTakumi-CPG	Itself	Inhibit TLR9 pathway	[90]
DNA nanoflower	CPG ODN	Itself	Activate TLR9 pathway; Stimulates immune cell proliferation	[100, 101]
	CPG ODN	Connect to DOX	Antitumor effect; Enhance aggregation in cells	[37]
	CPG ODN; shRNA ODN	Itself	Activate APC immune response antitumor effect	[103]
DNA dendrimer	Y-CPG	Itself	Activate TLR9 pathway	[109]
	Hexapod-tripod-CPG	Itself	Enhance macrophage uptake; TNF-α↑	[9, 36]
	Loop-CpG	Connect to TAT peptide	Enhance aggregation in cells; Activate TLR9 pathway	[111]

are easily degraded by nucleases to lose their functions. Therefore, DNA nanostructures cannot efficiently reach diseased tissues and organs when intravenously injected into vivo [115]. (2) DNA nanostructures lack targeted delivery methods, resulting in low cell absorption efficiency. Due to the strong electrostatic repulsion between the negatively charged cell membrane and DNA components, DNA nanostructures cannot easily enter the target cells [116], which limits the ability of diagnosing and treating certain types of diseases to hinder their practical application in vivo [117]. In order to make them possess target capability, DNA nanostructures are often modified with specific recognition ligands to upregulate cell receptors or cancer biomarkers, so that they can more effectively across cells through the receptor-mediated endocytosis via [54, 118]. Besides, because the methods which produce specific arrangement between ligands and DNA nanostructures in a precise and controlled manner are absent, the biological activity of incorporated targeting ligands is not significant [119–121]. (3) The limited drug payload capacity and size limitations of DNA nanostructures inhibit their therapeutic effects. For instance, the ratio of encapsulation between drug and ligand has the limitation of molecular pairing such as inserting Dox molecule into G/C bp instead of A/T bp, and their cell uptake rate is also affected by the optimal particle size (20 to 100 nm) of the nanocarriers in targeted tumor drug delivery [118, 122], displayed in weak drug loading capacity of nanosphere DNA nanocarriers which limited by particle size and drug loading. In this case, even if the nanoparticle drug delivery system is specifically internalized into diseased cells, the concentration of anticancer drug released from the nano-formulation cannot reach the therapeutic threshold, resulting in unsatisfactory therapeutic effects.

In clinical applications, DNA nanomaterials serve as double-edged sword. On the one hand, their nanoscale size lead them to penetrate biological tissues and may destroy biological functions [123]. On the other hand, if DNA nanomaterials with reasonable dose range can be completely removed and degraded in vivo, they will have great potential in the field of diagnosis and therapy [124]. Therefore, many studies focused on removing various nanoparticles in renal system [124, 125] and found that the filtration of nanoparticles through the kidney depends on many factors, including surface chemistry and the hydrophobic/hydrophilic nature of the nanoparticles [125]. To sum up, the current problems related to the application of nanoparticles in vivo mainly include the following three points: (1) the effective delivery of nanoparticles in vivo without causing damage to other tissues; (2) the balance between sufficient nanoparticle retention time in the body; (3) clearance of key components of nanoparticles in the body.

DNA nanostructures can not only be used as a vaccine to stimulate the immune response but also can reduce the immunogenicity by encapsulating surface proteins, to serve as a therapeutic agent of autoimmune diseases. The wire-frame DNA nano-octahedron (DNO) was encapsulated in PEGylated lipids resisting to nuclease digestion and injected into primary mouse splenocytes. Flow cytometry showed that the average fluorescence of spleen cells incubated with nonencapsulated DNO (N-DNO) was 111 ± 8 times higher than the average fluorescence of encapsulated DNO (E-DNO), suggesting that the spleen cells reduced the uptake quantity of DNA

nanostructures encapsulated in PEG lipid membrane [126]. Besides, DNA origami structure which was smeared with bovine serum albumin (BSA) found that the BSA coating can resist the degradation of endonuclease (DNase I) to significantly improve the stability of origami and enhance transfection of embryonic kidney cells (HEK293). Most importantly, the test also observed that the BSA coating attenuated the activation of immune responses in mouse primary spleen cells [127]. Therefore, surface packaging of DNA nanomaterials can suppress the body's immune response, showing the potential for treating autoimmune diseases.

Based on the activation of interferon gene (STING), it is effective to increase the production of innate and adaptive immunomodulatory proteins such as CXCL10 and TNF-α undergoing the transcription factors interferon-regulatory factor 3 (IRF3), nuclear factor-kB (NF-κB), and Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) pathway [128]. A novel immunomodulatory molecule called cyclic GMP-AMP (cGAMP) which is an agonist of STING began to bring itself into notice. Tan YS et al. delivered cGAMP into the tumor cells of head and neck squamous cell carcinoma by loading in nanosatellite vaccine, resulting in enhancement of tumor antigen density and powerful and specific antitumor effects [129]. However, the STING ligand DMXAA may induce an unwanted type II immune response when activating the STING-TBK1-IRF3 pathway [130]. In order to deal with the stimulation of type II immune response by STING, there was a study combining 3′3′cGAMP and K3 CPG weakly inducing interferon alone [7, 131] to jointly stimulate the immune response of cells. The compound was demonstrated that could synergistically induce NK cells to produce IFN-γ through the synergistic effect of IL-12 and type I interferon. And further research evaluating the influence of compound in vivo demonstrated it could suppress the type II immune response while inducing strong type I immunization and CTL response [132]. Thus, some unconventional vaccine adjuvants can also be combined with DNA nanoparticles according to the desired immune effect and be used with CPG to offset the adverse effects by agents in a certain immune link while amplifying the specific desired immune link.

More newly developed DNA nanostructures have opened a new path for the development of DNA vaccines. Bead-chain DNA nanowires (BS-nanow) is assembled from DNA tetrahedron units with precise nanometer-scale spatial control, capable of accommodating chemotherapeutic agents with high payload capacity (1204 binding sites) as well as possessing a 60-fold enhanced binding affinity for target cells. Although its application in immunoengineering is rarely explored, its high load capacity, targeted localization ability, programmability, and biocompatibility make it have immunoengineering potential, especially in the field of vaccine preparation [133]. With the development of recombinant DNA technology and biocomputer technology, it is believed that more DNA nanostructures will be developed and costs will gradually be reduced. The "plug and play" of DNA nanostructures as vaccine vectors can be realized, and even intelligent DNA can be manufactured to build artificial immune defense system.

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