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Research on Nano-drug Delivery System in Tumor Immunotherapy

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Abstract: *Cancer poses a severe threat to human life and health. In recent years, immunotherapy has been widely used in cancer treatment. However, easy recurrence, drug resistance, and side effects have become urgent issues to be solved. Nano-drug delivery systems loaded with anti-tumor drugs have many advantages, including powerful targeting, high safety, and low side effects. Based on this, applying nano-drug delivery systems in immunotherapy is a research hotspot. This paper reviews the application status and research progress of nano-drug delivery systems in tumor immunotherapy. The history and types of nanocarriers and the current status of bispecific nanobodies and nucleic acid drugs are presented.*

Keywords: *Nano-drug delivery system; Tumor immunotherapy; Bispecific nanobodies; Bacterial carrier; Nucleic acid drug*

1. Nano-drug Delivery System

The nano-drug delivery system refers to the delivery of drugs to target cells through nanocarriers or nanocarriers as nanodrugs. Nano-drug delivery systems can deliver drugs in a targeted, efficient, and low-toxic way. With the increasing use of tumor immunotherapy, precise drug delivery and release is becoming increasingly important. Nano-drug delivery systems are used in tumor immunotherapy due to their characteristics. Drug delivery is required in the initial screening, animal experiments, and clinical drug development trials, as shown in Figure 1. The development of the nano-drug delivery system has gone through three generations and is currently in the embryonic stage of the third generation, as shown in Figure 2.

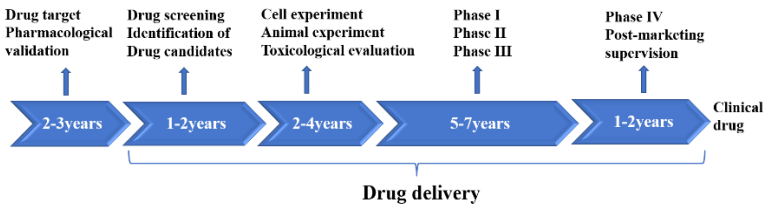

Figure 1: Drug delivery in the drug development process

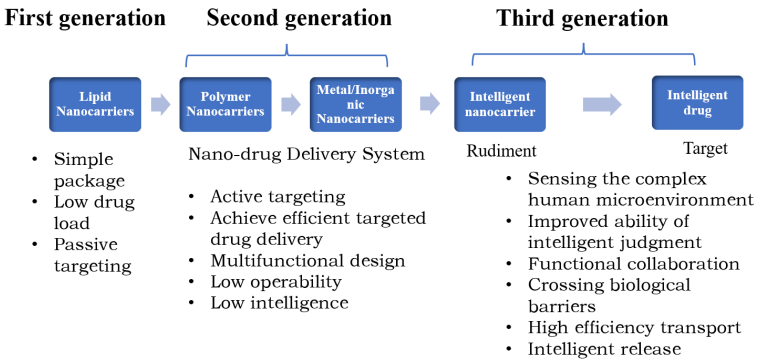

Figure 2: Development of nano-drug carrier

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2. Classification and Preparation of Nanocarriers

2.1 Lipid Nanocarriers

Liposomes are lipid microcapsules with an inner aqueous phase and an outer phospholipid bilayer and are widely used as hydrophilic drug carriers. It has the characteristics of non-toxicity, non-immunogenicity, and high bioavailability, which can prolong the half-life of drugs, enhance drug targeting, and easy degradation in living bodies. Lipid nanoparticles (LNP) are currently divided into solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). The diameter of SLN particles is between 50nm-1000nm, which has the advantages of protecting the internally sensitive drugs from degradation, controlling the release rate of drugs, and high physical stability. However, the shortcomings are limited drug loading capacity and easy leakage of drugs. NLC is a mixture of solid lipids and liquid lipids, which can increase drug loading and encapsulation efficiency. Tran Tuan Hiep et al. prepared artesunate nano-lipid carrier (ART-NLCS); the analysis confirmed that compared with free artemisia Jayesh and other succinates, ART-NLC significantly enhanced the killing effect of human breast cancer MCF-7 and MDA-MB-231 cells in vitro, and significantly increased the apoptosis rate of MCF-7 and MDA-MB-231 cells in vivo [1]. Lipid nanoparticles (LNP) are currently the primary non-viral delivery system for nucleic acid-based therapy, including siRNA, mRNA, or plasmid DNA that treats disease by silencing pathogenic genes or expressing therapeutic proteins. In addition, siRNA LNP may selectively damage fusion genes expressed only in cancer cells, thus constituting a tumor-specific target [2]. With the development of detection technology, the composition and formation mechanism of LNP have been explored. For example, Jayesh A. et al. used cryo-transmission electron microscopy (cryo-TEM) and small-angle X-ray diffraction to demonstrate the structural characteristics and formation mechanism of LNP-siRNA. In the structure of the LNP-siRNA system, siRNA is located between the lipid bilayer, and the middle is the hydrophobic central oil phase separated by cationic lipids [3]. To realize the full potential of LNP, further optimization of its formulation and surface modification is required. L.E. Swart et al. described a safe and simple method for LNP-siRNA modification [4]. This method combines the copper-free click method with the post-insertion strategy. The results showed that this method successfully modified LNP-siRNA with fluorescent labeling and targeting ligands.

2.2 Polymer Nanocarriers

Polymer nanocarriers are prepared by a simple process from synthetic polymer materials or natural macromolecular materials. The advantages of polymeric nanocarriers include good structural stability, high encapsulation efficiency, and drug loading, and protection of drugs from enzymes and body fluids. A series of block polyester polymer materials (PEG-PLA, PEG-PLGA, PEG-PCL) can be obtained by ROP reaction with different monomers (lactide-LA, glycolide-GA, caprolactone-CL) using PEG as initiator [5]. These materials have amphiphilic structures, have good biocompatibility and biodegradability, and are suitable drug carriers. Studies have shown that nanoparticles based on such polymer materials can effectively load anti-tumor drugs with low bioavailability and improve drug stability. In addition, it allows long-term circulation, targeted drug delivery, and controlled release of drugs into the body, thus enhancing tumor healing and reducing adverse reactions to normal tissues [6]. Polymeric nanomedicines have made significant breakthroughs in clinical trials. South Korea's Genexol-PM® and Russian Paclical® have been clinically approved. They are all polymer nano drug preparations of paclitaxel. Genexol-PM® is prone to drug burst release and low bioavailability.

At the same time, polymers as carriers pose many problems. The physical adsorption between the drug and the polymer is not strong, and the effect of the passive target transport of the drug obtained by the EPR effect is unclear. Therefore, there is an urgent need to improve the stability of nanomedicine during in vivo transport, improve the targeting ability of polymeric nanoparticles, and achieve precise drug release. For the above problems, researchers have designed and synthesized PEG-polyester polymers with novel structures or chemically modified methods to obtain novel multifunctional PEG-polyester polymeric drug carriers by taking advantage of polymer nanocarriers' modifiable properties. Kai et al. linked PEG and DOX N-(1,3-dihydroxypropan-2-yl) methacrylamide (DHPMA) through a disulfide bond to form the nanopolymer pDHPMA-DOX-SS-mPEG [7]. It enters 4T1 cells through a variety of endocytic pathways. The experimental results showed that pDHPMA-DOX-SS-mPEG exhibited pH and enzyme responsiveness and improved antitumor efficacy in 4T1 tumor-bearing mice. Zhang et al. prepared PEG-PLA nanoparticles co-loaded with antitumor drug paclitaxel (PTX) and anti-angiogenesis drug itraconazole (ITA) [8], compared with nanoparticles loaded with PTX; the introduction of ITA improved the stability of the carrier. Studies have shown that PTX/ITA co-loaded

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nanoparticles can inhibit tumor cell proliferation in treating non-small cell lung cancer and improve PTX resistance. Researchers have recently used pH sensitivity to achieve precise drug delivery to target cell sites. A biodegradable polymer mPEG-PCL has been synthesized. In the presence of curcumin, methotrexate (MTX)-conjugated mPEG-PCL (MTX-mPEG-PCL) was self-assembled into micelles by nanoprecipitation. The tumor release rate was tested at different pHs to discover the most suitable pH for stable drug release [9].

2.3 Nanoemulsion

Nanoemulsion is a transparent or translucent oil-water dispersion composed of tiny oil droplets uniformly dispersed in an aqueous medium. The core of the nanoemulsion is the oil phase, the surface has a hydrocarbon chain, and the water solubility is good. Studies have shown that the benefits of nanoemulsions lie in improving the solubility and bioavailability of insoluble drugs. Some scientists have used gelatin and ethoxylated monoglyceride to prepare curcumin nanoemulsions and observed growth inhibition and apoptosis of pancreatic cancer cells. They developed a stable, safe, and efficient dosage form of anti-pancreatic cancer drug [10].

2.4 Metal/Inorganic Nanocarriers

Inorganic nanocarriers mainly refer to nanocarriers constructed by inorganic materials (such as mesoporous silicon, gold, and iron), which have the advantages of simple preparation, easy modification, and small molecules. Because molecules are small, they can penetrate low-permeability tumors such as pancreatic tumors. As a result, it is the development direction of inorganic nanocarriers, which can enter dense tumors by their advantages. Zhang et al. grafted ruthenium (II) polypyridine complexes on gold nanospheres; optically active ruthenium (II) complexes can improve the near-infrared absorption of particles for real-time luminescent imaging in living cancer cells to guide cancer photothermal therapy [11].

Stoddart et al. covalently linked ruthenium (II) dipyridyl phenazine (Ru-dppz) complexes to the surface of mesoporous silica nanoparticles (MSNPs), and filled the pores with paclitaxel to help kill cancer cells [12].

2.5 Engineered Exosomes

Exosomes are natural bilayer nanoparticles generated by eukaryotic cells, with a diameter of about 30-150 nm. It is an extracellular vesicle structure secreted by cells, including bioactive molecules and nucleosome-derived components such as mRNA, miRNAs, cDNA, lipids, and proteins. [13]. According to the reports, there are about 41860 proteins, 7540 RNAs, and 1160 lipid molecules [14-16]. Exosomes are widely present in all body fluids and tissues, including plasma, urine, saliva, milk, amniotic fluid, cerebrospinal fluid, and lymph. [17] Exosomes play a role in communication, and donor cells transport exogenous substances such as proteins, mRNAs, and miRNAs to recipient cells via exosomes. Therefore, this natural nanocarrier has been used for drug delivery [18]. As a drug carrier, exosomes have low toxicity and good tissue tolerance. Furthermore, it is a natural nanocarrier with higher biocompatibility. The structure contains a phospholipid bilayer, so ribonuclease is difficult to degrade. It is small in diameter and can even cross the blood-brain barrier (BBB). The exosome carrier can significantly improve the drug load and maintain the blood drug concentration at a high level.

As an excellent natural carrier, exosomes can be loaded with various drugs to complete targeted therapy. Loading methods are divided into in vivo preload and in vitro loading, and most exogenous drugs use an in vitro loading strategy. In vitro loading is divided into two types: active and passive. Electroporation is a standard passive packaging method. Siqing et al. developed an exosome-functionalized intraocular lens loaded with doxorubicin (Dox) using the homologous targeting and high biocompatibility of exosomes. Electroporation loaded dox to effectively prevent posterior capsular opacification (PCO) [19]. To give specificity for targeting exosomes, we engineer them because they are superficially easy to modify. Modification strategies are divided into genetic engineering and chemical modification. Genetic engineering is practical for proteins and polypeptides but only for those whose gene sequences can be encoded; chemical modification has a broad spectrum and can be modified by coupling reactions and lipid assembly. Tanziela et al. loaded biologically assembled AgNC in cancer exocytosis exosomes. They found that engineered exosomes showed higher cell accumulation and could be used as intelligent cancer-targeting agents to selectively attack cancer cells. Therefore, engineered exosomes can be used for bio-responsive self-assembly of intelligent drug carriers in targeted

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chemotherapy [20].

2.6 Bacterial Carrier

In the medical works of the ancient Egyptians, it is recorded that using bacteria to infect tumors can reduce tumor size. In the mid-19th century, William Coley, the father of cancer immunotherapy, used "Coley's toxin" to treat cancer. FDA approved the use of BCG in treating superficial bladder cancer in 1990. The development of genetic engineering, synthetic biology, and material science has also led to the vigorous development of the field of bacterial-mediated tumor therapy.

There are a variety of microbial communities in the human body, and the complex interaction between the human body and microorganisms plays a crucial role in human health and disease. For example, in the intestinal flora of the human body, the imbalance of the microflora will adversely affect the epithelial cells and induce colon cancer. Zhang et al. engineered glycosylated nanoparticles to avoid affecting gut flora and promote targeted antibiotic absorption [21].

Studies have shown that facultative anaerobes can effectively target deep areas of tumors. The blood vessels of the tumor tissue are complex, highly hypoxic, easy to enter, and challenging to get out, which promotes bacterial invasion. Many inflammatory factors in the tumor microenvironment are rich in nutrients and have a certain predisposition to bacteria. Moreover, the immunosuppressive microenvironment in tumor tissue prevents bacteria from being cleared by the body's immune system.

The bacteria can be used as an anti-cancer drug to destroy tumor cells. It can also be used as a drug carrier to efficiently present antibodies, mRNAs, protein drugs, and oncolytic viruses to precisely target and colonize the tumor region.

Escherichia coli (EcN) can be used as a suitable drug carrier because of its non-toxicity, tumor targeting, and regulation of human immunity. Some studies have demonstrated that recent applications of EcN as a carrier include the delivery of immunotherapeutic nanobodies containing the proteins CD47, programmed death-ligand 1 (PD-L1), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [22-23].

The dose-dependent side effects of bacterial therapy have become a bottleneck in the field. To solve this problem, many researchers have engineered bacteria to reduce toxicity. The genome information of many bacteria has been successfully decoded, so the engineering transformation and modification of Escherichia coli and Salmonella typhimurium has become the current research hotspot. The engineered Salmonella typhimurium strain that secretes Vibrio vulnificus flagellin B (FlaB) has shown good immunotherapeutic effects in a mouse tumor model [24].

The main limitation of bacterial therapy is that the relationship between the drug's optimal dose and the target disease's pharmacodynamics is unclear, and little is known about the pharmacokinetics of bacteria in vivo. Long-term stable bacterial colonization at the tumor site, reduced dose, reduced toxicity and side effects, and improved safety are essential for further research on live bacterial therapy.

Bacterial outer membrane vesicles (OMV) are vesicles with a diameter of 20-250 nm derived from the outer membrane of bacteria. Most gram-negative bacteria, such as Escherichia coli, Salmonella, and Helicobacter pylori, produce OMVs. OMVs have many biological components, including bacterial antigens and binders. It has intrinsic immune-boosting properties and can carry a variety of cargoes. Therefore, OMVs have been designed to treat various human diseases, such as tumor vaccines, adjuvants, immunomodulators, drug delivery systems, and antimicrobial binders. It has become a research hotspot in the field of tumor vaccine to isolate the membrane antigen of tumor cells from the cell membrane of bacteria as an adjuvant and to mix the two to make a hybrid vaccine. Researchers can make plug-and-play personalized vesicle vaccines. Various OMV-based vaccines against pathogens have been developed in animal models, such as Escherichia coli and Salmonella enteritidis [25-26].

3. Novel Nanomedicines in Tumor Immunotherapy

In tumor immunotherapy, bispecific nanobodies have become a current research hotspot due to the limitations of monoclonal antibodies in the population. Affected by the COVID-19, nucleic acid drugs have been developed rapidly. Both types of drugs require nano-drug delivery systems to complete drug delivery. They are both nanomedicines and nanocarriers.

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3.1 Bispecific Nanobodies

Bispecific nanobody (BsNb) can simultaneously recognize two different antigens or epitopes of the same antigen, with versatile target combinations and diverse mechanisms of action. Therefore, bispecific antibodies are an essential part of antibody drug development. Compared with conventional nanobodies, they have more substantial specificity and targeting and fewer side effects and toxicity, which suggests the advantage of reducing immune escape and drug resistance during treatment. According to the action sites, it can be divided into four types, as shown in Figure 3.Table 1 shows the current clinical and preclinical bispecific antibodies.

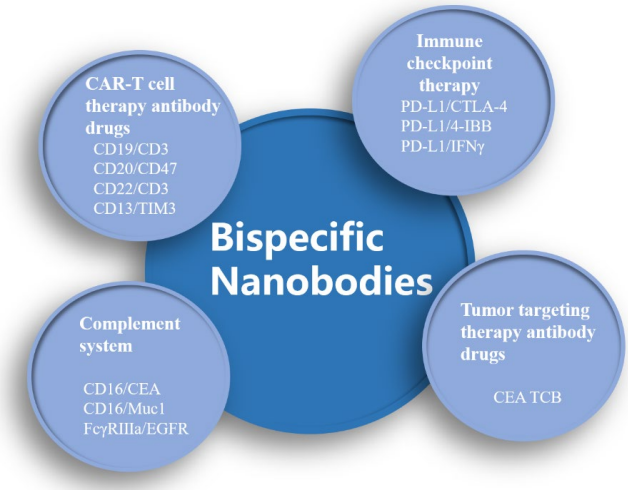

Figure 3: The application of bispecific nanobodies in tumor immunotherapy fields

Table 1: Anti-tumor bispecific nanobodies

Antibody name	Target	Disease to treat	Years	References
RR2-H-RR4-Lip	HER2 epitope 1 / HER2 epitope 2	Breast cancer	2018	[27]
Muc1-Bi-1	Muc1/CD16	Cancer caused by abnormal expression of Muc1 cells	2018	[28]
NanoCAR	HER2 /CD20	B-cell leukemia and lymphoma	2018	[29]
PEG-S-Fab	CEA/CD3	Human Colon Adenocarcinoma Therapy	2018	[30]
SBC77	CEA/CD16a	Treatment of CEA Overexpression in Tumors	2019	[31]
Bi1 /Bi2	FP /EGFR	Lung cancer and other malignant tumors with high expression of EGFR	2020	[32]
TSsdAb	CD16 /EGFR	EGFR positive tumors	2020	[33]
PD-L1/TIGIT	PD-L1/TIGIT	Locally advanced or metastatic non-small cell lung cancer	2020	[34]
CD96/PD1	CD96/PD1	Did not enter the clinical trial	2022	[35]

3.1.1 Antibody Drugs for Immune Checkpoint Therapy

PD1 is a classical immune checkpoint on the surface of T cells. It interacts with the tumor cell surface ligand PD-L1 and inhibits T-cell activation. Studies have shown that PD1 monoclonal antibodies can effectively block the binding of PD1 to tumor surface ligands and exert anti-tumor effects in various malignant tumors. PD1 antibody drugs opened the era of immune checkpoint therapy antibody drugs. However, monoclonal antibodies are often resistant and effective in a small number of patients, which limits their clinical application. Bispecific antibodies can solve these problems, so finding new target combinations for combination and making bispecific antibodies is an urgent problem. Corning Jireh obtained the nanobodies KN035 and KN044 targeting PD-L1 and CTLA-4 by immunizing camels to construct bispecific nanobodies that can simultaneously bind PD-L1 and CTLA-4 [36-37]. Shanghai Luoqi Pharmaceutical [38] constructed an anti-PD-L1/4-1BB bispecific nanobody, limiting immune activation to the target antigen tissue and reducing toxicity. In structural design, bispecific antibodies targeting immune checkpoints do not require antibody-dependent cell-mediated cytotoxicity (ADCC).

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Therefore, the Fc backbone of bispecific antibodies mainly adopts the Fc backbone of silent IgG1 or the Fc backbone of IgG4 with weak ADCC effect. The bispecific design of nanobodies by genetic engineering has become a research hotspot in tumor immunotherapy. Tilman Schlothauer et al. found two newly engineered hIgG Fc domains [39], hIgG1-P329G LALA and hIgG4-P329G SPLE, which can eliminate the interaction between FcγR and C1q and produce IgG molecules with complete effect silencing.

3.1.2 CAR-T Cell Therapy Antibody Drugs

CAR-T cell therapy is a new type of tumor immunotherapy that enables T cells to express tumor-specific chimeric antigen receptors through gene modification. Although CAR-T cell therapy has a clinically significant effect on malignant B-cell tumors, it is not sufficient for other solid tumors. To overcome the limitations of current CAR-T cell therapy, the researchers are committed to finding other antigen-binding domains that can replace single-chain antibodies, among which bispecific nanobodies are ideal. The research on nanobodies as CAR extracellular antigen recognition domains mainly targets CD19, CD20, CD30, and CD22. Ma et al. constructed the nanobody fusion protein HuNb1-IgG4 and constructed the anti-CD47/CD20 bispecific nanobody by the combination of HuNb1 and Rituximab, which showed more vigorous anti-tumor activity in vivo [40]. CD20-targeted T cell-involved bispecific antibody Glofitamab can induce durable complete remission in relapsed or refractory B-cell lymphoma: it has entered phase I clinical trials [41].

3.1.3 Antibody Drugs Based on Complement System-mediated

The complement system is capable of removing pathogens and damaged host cells. However, only some therapeutic antibodies can induce complement-dependent cytotoxicity. We need to find new strategies to enhance complement activation. The complement system is closely related to the immune escape of tumors, mainly including complement intrinsic components C3 and C1q, complement activation products C3a and C5a, and complement regulatory protein H factors and mCRPs. Li et al. [42] constructed a humanized bispecific nanobody that simultaneously targets the NK cell FcγRIIIa receptor (CD16) and EGFR dimerization interface and has anti-tumor activity. Pederser et al. constructed a bispecific nanobody that simultaneously targets Properdin (FP) and cancer epidermal growth factor receptor (EGFR) [43], providing a novel strategy for direct activation of the replacement supplement. Andreas et al. conducted a phase 2 clinical evaluation of CD3/CD19 bispecific antibody, which can prolong the survival of patients with relapsed/refractory diffuse large B-cell lymphoma [44]. Complement-mediated drugs are more effective when administered subcutaneously.

3.1.4 Antibody Drugs for Tumor Targeted Therapy

Carcinoembryonic antigen T-cell bispecific antibody (CEA TCB) is a novel IgG-based T-cell bispecific (TCB) antibody for the treatment of solid tumors expressing CEA. It is currently in phase I clinical trials [45].

3.2 Nucleic Acid Nanomedicines

With the development of vaccines for COVID-19, mRNA drugs have received extensive attention. Moderna and Pfizer have produced mRNA vaccines in 2020. Figure 4 is the development history of mRNA drugs. However, mRNA can be used not only for vaccines but also for diseases at the protein level. Tumor mRNA offers broad prospects for the development of personalized tumor vaccines. At present, LNP system delivery is mainly used to interfere with the tumor microenvironment, trigger an immune response in tumor tissues, and play a role in the destruction of tumors. LNP nanocarriers are mainly prepared by the microfluidic method.

The current challenges for mRNA drugs are how to avoid unnecessary immunogenicity, prolong the duration of action, and avoid systemic antibody distribution through drug administration. Applying AI to the molecular design of mRNA drugs will be the future development direction.

The mRNA needs a stable delivery system. Due to the poor stability of naked mRNA, short in vivo half-life, easy removal and negative charge, and low cell penetration efficiency, nanoparticle carriers can prolong the circulation time in vivo and enable targeted drug delivery.

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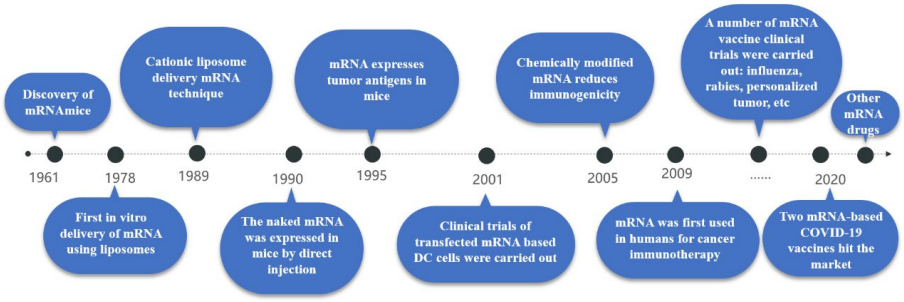

Figure 4: mRNA Drug Phylogeny

4. Summary and Prospect

Nano-drug delivery systems solve the problems of targeted drug delivery, precise release, and reduction of side effects and toxicity. However, factors limiting its development also include clear individual patient differences, limitations of animal models, unclear tumorigenesis mechanisms, and great controversy over the universality of the EPR effect. The research and development are in the early stages of the third generation of intelligent nanocarriers. Scholars are constantly exploring new properties and new functions different from traditional dosage forms so that intelligent nanomedicines have the characteristics of controllable self-assembly, intelligent modification, and multi-function. Such intelligent nanocarriers are called intelligent nanorobots. For example, in recent years, selective response nanorobots have been designed and prepared by using DNA origami. In addition, AI technology can be used to synthesize nanocarriers.

Considering the individualized differences of patients, the functional integration strategy of nanosystems can be combined with personalized medicine as a novel strategy. We choose the appropriate delivery system, drug combination, and treatment combination depending on the patient's genes. To further explain the formation mechanism of various tumors, clarify the basis of tumor growth - the characteristics of the tissue composition of the tumor microenvironment, and find innovative points from the pathogenesis of the disease. Don't just cure the symptoms but also the root cause and find new targets. Based on this, new nanocarriers were developed.

Research and development of nanocarriers is no longer a single discipline but tends to be a combination of multiple disciplines, including pharmacy, genetic engineering, synthetic biology, and material science.

We are convinced of the results of our experiments, strongly believe in our opinions, and avoid going with the flow. Giving up because the results are unsatisfactory and moving to another field is also improper. A vast brain, different thinking, and cross-integration will lead to different results.

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