# Exploring liposome-targeted chemotherapy combined with immunotherapy in cancer

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# 01
# General introduction

# Introduction



Cancer is a global health problem that severely impairs quality of life and life expectancy. Cancer is not one disease but can occur in diverse appearances, as virtually all cell types of our body can transform into the malignant form. This transformation is generally a very rare event which can lead to abnormal cell proliferation and metastasis resulting in severe pathology and even death. Several therapies have been developed to clear malignant cells from the body however often recurrences occur after initial treatment, which raises patient mortality rate significantly [1]. In the clinic, the main treatments are surgical resection, radiation therapy, chemotherapy, and immunotherapy [2–6]. Among these strategies, chemotherapy primarily acts by interfering various metabolic processes involved in tumor cell proliferation, producing effective therapeutic effects, but also has many drawbacks, such as drug resistance, short in vivo circulation time, poor tumor permeability, and cytotoxic effects on normal healthy tissues [7–11]. The genetic diversity of solid tumors often limits the effectiveness of chemotherapy drugs and causes drug resistance [12]. The drug resistance of solid tumors can source from many mechanisms, including the (hyper)activation of drug efflux pumps and DNA repair dysfunction, which lead to de novo gene mutations and subsequent changes in the upstream drug metabolism pathways [13]. For example, many anti-cancer drugs cannot achieve effective results because of their limited penetration into tumor tissues [14,15]. The poor permeability of tumor tissues caused by high tumor stromal pressure and complicated interaction between stromal cells and tumor cells increase the inaccessibility of therapeutical agents [16]. Therefore, the tumor microenvironment (TME) and other obstacles that limit drug delivery have received great attention, and it is crucial to develop rational drug delivery system to maximize anti-cancer efficiency as well as minimize adverse effects.

## 1. Cancer nanomedicine

Cancer nanomedicine focuses on nanoparticle-based therapy and aims for the improved activity of anti-cancer drugs and the decrease of adverse effects [17]. The vehicle to deliver anti-cancer drugs, namely nanoparticle or nanocarrier, have unique properties such as nanoscale size (particles with average diameter of 1-999 nm), high surface-to-volume ratio, and favorable physio-chemical characteristics [18]. With the advancement in material properties, cancer biology, as well as nanotechnology, cancer nanomedicine gains high-speed development and becomes more sophisticated where multiple purpose is achievable [19]. First, nanoparticles can improve bioavailability of anti-cancer drugs with poor solubility and chemical stability. A typical example is Abraxane®, albumin-nanoparticles-bound Paclitaxel, shows increased solubility and improved accumulation in tumor sites [20]. Secondly, nanocarriers are capable of protecting anti-cancer agents from rapid clearance before entering to the target site and thereby affect their pharmacokinetic profile. For example, in vivo enzymatic cleavage of nuclear acid and protein products can be prevented when encapsulated into nanocarriers or coupled to synthetic polymers [21,22]. Thirdly, with the employment of nanoparticles, improved biodistribution and accumulation in desired tissue or organ can be achieved by both prolonged circulation and targeted strategies. Rational construction of nanoparticles benefits drug penetration and biodistribution of chemotherapy or targeted compounds to a specific area, such as tumor cells and/or their surrounding stromal environment. Fourthly, nanocarriers can be designed to release payload upon certain stimuli (light, sonics, pH, temperature. etc.) [23,24], enabling increased internalization and controlled intracellular drug release. Furthermore, targeted nanomedicine may reduce drug resistance generated by drug transporters, which facilitate the efflux of drugs out of cancer cells [25]. For example, doxurobicin liposomes modified with phase fusion protein for specific targeting avoided P-glycoprotein-mediated drug expulsion and increased the cytotoxicity of payload in pancreatic cell line [26]. Last, but not least, nanocarriers exhibit potential for integration as imaging or diagnostic

tools [27,28], combining several regiments simultaneously [29,30], and regulating the tumor microenvironment [13,31,32].

*1.1 Typical nanoparticles for cancer therapy*

Over time, multiple types of nanoparticles have been used for clinical use or tested in clinical trials. Based on the materials and composition, there are three main types of nanoparticles: organic, carbon-based, and inorganic. The organic nanoparticles are made from carbohydrates, lipid, polymers, proteins or any other organic compounds. The most frequently used examples are drug conjugates, lipid-based nanocarriers [33,34], and polymer-based nanocarriers [35]. They are normally low or non-toxic, biocompatible, and have high potential to encapsulate payloads. Carbon-based nanoparticles are solely made of carbon atoms, e.g. C60 fullerene and carbon quantum dots. Inorganic nanoparticles are nanoparticles that are made from metal, ceramic, etc [32,33]. Their stable structure and high surface area make them suitable for drug delivery, tissue engineering, and diagnostics. Practically, all these nanoparticles can be designed to deliver therapeutic nucleic acids, immunotherapeutic drugs, or chemotherapeutic agents to tumors in a targeted or non-target manner. Below I mention a few commonly used nanoparticles in cancer therapy with specific advantages and applications are briefly introduced:

1) Drug conjugates: one of the most successful nanomedicine therapeutics in clinical use. They are comprised of two parts, active agents and targeted antibodies (or polymer), which are covalently connected. The conjugate is usually mono- or oligomeric, intended to improve targeted delivery of the drug without necessarily impacting on their physio-chemical properties. There are different antibody drug conjugates (ADCs) on the market, such as brentuximab and trastuzumab. For example, upon coupling with anti-CD30 antibody, brentuximab redirects itself to CD30+ cancer cells selectively and thereby leading to much less toxicity in patients with Hodgkin's lymphoma [36].

2) Lipid-based nanoparticles (LNP): owning to their unique structure, they have significant advantages for combination therapy. The most frequently investigated are liposomes, lipoplexes, and ionizable LNPs. Liposomes were the first developed closed bilayer phospholipid systems with clinical approval for cancer treatment. They have attracted much attention due to their excellent biocompatibility, efficient drug delivery, and the ability to protect biologics from degradation. More details will be described in Chapter 2. Lipoplexes are commonly used for non-viral gene delivery, and their unique inherent properties, such as high stability, enable them to effectively improve cellular uptake of drugs with low cytotoxicity [37]. Similar to lipoplexes, ionizable LNPs are attracting recent attention due to their high transfection and encapsulation efficiency, and the ability to avoid degradation of RNA products.

3) Micelles: helping drugs escape uptake by the reticuloendothelial system while maintaining prolonged circulation times. The amphiphilic structure of micelles allows them to encapsulate hydrophobic and anchor hydrophilic drugs efficiently, providing a platform for cancer therapy [38–40]. Because their chemical versatility, micelles comprising of synthetic polymers are widely applied in treatment (e.g. prodrug strategy) as well as diagnostics, both in preclinical and clinical investigations.

4) Inorganic nanoparticles: stable platform with high specific surface area to improve the efficiency of conventional medicine. The unique optical, electrical, catalytic, magnetic and other properties of inorganic nanoparticles also make them emerge as frontrunners in the field of cancer therapy [41,42]. Novel inorganic nanoparticles such as nano-diamond [43] graphene [44] have received considerable attention for cancer therapy.

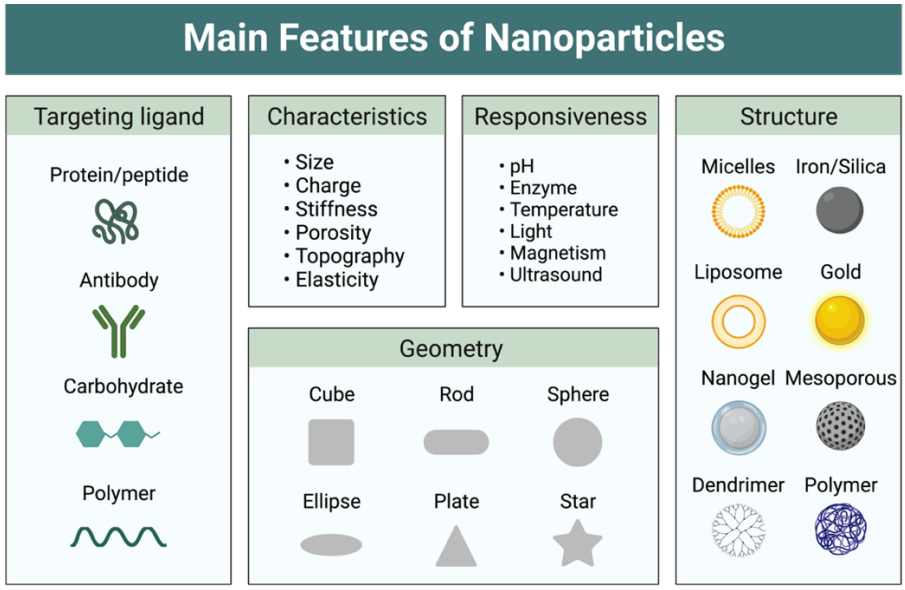


**Fig. 1. The major physicochemical properties that influence the delivery of nanoparticles.** A broad spectrum of physicochemical properties of nanoparticles influences the journey of them to the target site. Structure size, shape, rigidity, charge, and surface chemical composition, etc., are the predominant factors affecting the outcome of biodistribution, therapeutical effect, as well as immune modulation. (Created with BioRender.com)

*1.2 Key physiochemical properties of nanoparticles*

To gain a good biodistribution and anti-cancer effects, it is crucial to have a precise understanding of the interrelation between nanoparticles' physiochemical properties and their surrounding environments. Typically, there are several properties influencing the delivery of nanoparticles to the site of interest and further biological activity (Fig. 1). These nanoparticles can be designed with various materials and have different physical properties including size, geometry, surface charge, porosity and elasticity. They can also be functionalized with multiple ligands for either enhancing targeted ability or prolonging circulation time. These properties play a crucial role in how nanocarriers cross biologic carriers on their travel to a tumor.

### 1.2.1 Size

As one of the most important features of a nanoparticle, appropriate size allows nanoparticle to pass through different biologic barriers in the body. It significantly affects the blood circulation, cellular uptake, cytotoxicity, tumor penetration and clearance [45,46]. It is generally believed that particles with small size are internalized by cells faster than those with large size, which contributes to a high uptake of payload within the target cells and tumor tissue. However, this does not mean the smaller the nanoparticle, the better the anti-cancer effects. Instead, a certain range of size should be achieved for an efficient delivery, based on toxicity and clearance aspects. It has been shown that smaller nanoparticles also bring higher toxicity than larger ones. For example, silver nanoparticle synthesized with different size (10, 40, 40, 75 nm) displayed size-dependent cytotoxicity in human lung cells, where 10 nm particles influenced the cell viability the most [47]. In addition, nanoparticle with a diameter less than 10 nm are more likely to be eliminated rapidly by the kidneys [48], while particles bigger than 200 nm may activate the complement system and lead to their rapid clearance from blood [49]. Based on these observations, an optimal size for nanoparticles should be designed individually according to the practical needs (cellular uptake, biodistribution and clearance from the organism).

### 1.2.2 Shape

The shape of nanoparticles has an impact in cellular internalization, biodistribution, and anti-tumor effects [50,51]. Nanoparticles can be synthesized in different shapes, including cubes, rods, spheres, etc. (Fig. 1). When nanoparticles get close to target cells, the contact angle or area initially occurs and subsequently dictate the efficiency of internalization [52]. For example, compared to spherical nanoparticles, rod-shaped nanoparticles have a larger contact area for receptors on the membrane, leading to less receptor available for binding. Also, the membrane wrapping time required for nanoparticles influence their uptake and further cargo release [53]. In this

scenario, cylindrical particles require more time to be internalized completely than spherical ones. It is reported that spherical nanoparticles exhibited the highest uptake, followed by cubic, rod- and disk-like nanoparticles [54]. Moreover, it has been found that spherical nanoparticles also displayed relatively lower toxicity than rod-shaped nanoparticles [55].

### 1.2.3 Charge



Along with size and shape, the charge of nanoparticles could also affect their interaction with biomolecules and cell surface [56,57]. Due to the electrostatic repulsive force between cell membrane and anionic surface of nanoparticles, negatively charged nanoparticles binds less effectively to cells than neutral and positively charged particles. However, the surface charge also impacts their toxicity properties in biological environments. The anionic surface of these nanoparticles contributes to their safe application in vivo, whereas cationic nanoparticles bind to various biomolecules/cells in the circulation and show more side effects [58]. In the clinic, ionizable nanoparticles have been developed to carry a safe and efficient delivery. The most famous example is the vaccine for COVID19, where ionizable lipid such as SM102 or ALC-0315 is employed to grant a neutral particle in the physiological environment but cationic particle in acidic pH. In addition, it seems hepatic clearance can be influenced by surface charge. Nanoparticles with high negative or positive charge tend to be cleared faster by liver Kupffer cells from blood circulation, compared to neutral nanoparticles [59,60].

### 1.2.4 Surface modification

Surface chemical modification is pivotal to offer stability and selectively of nanoparticles in the physiological medium. Upon injection, nanoparticles face rapid clearance by the reticuloendothelial system, which promotes the application of polyethylene glycol (PEG) in the pharmaceutical field. The hydrophilic PEG can mask nanoparticles and decrease unwanted protein absorption (stealth effect), which reduce phagocytic uptake and prolong the

half-live of nanoparticles [61]. PEGylated nanoparticles generally show longer circulation in blood and higher tumor accumulation than non-PEGylated nanoparticles. Moreover, much experimentation has gone into exploring the surface functionalization to reduce non-specific cell uptake and gain better anti-tumor effects. Multiple ligands can be anchored to the surface of nanoparticles to achieve high selectivity, such as proteins, peptides, antibodies, carbohydrates, and polymers etc. The targeting efficiency is related to the density of ligands on the nanoparticles, the receptor expression on the cell surface, and tumor penetrability.

### 1.2.5 Responsiveness

Apart from being stable in the circulation, it is also recognized that timely release of payloads upon target site can improve therapeutical outcomes. There are two main classes of triggers under investigation, internal triggers that are intrinsic to the disease site such as enzymes, pH, and glutathione, and external triggers from human manipulation such as heat, ultrasound, light, and magnetics. Normally nanoparticles can be designed to transform their structure and/or release their cargos in enzyme rich, low pH environment through the use of some enzyme- or pH-sensitive materials [62]. However, the distribution of these triggers may limit their efficacy. With the growing needs of precise medicine and combinational therapy, external triggers were explored much in the past two decades. These external stimuli can be controlled precisely on the location, period and intensity, which offer more opportunities for multiple therapeutical purposes. For example, ultrasound-responsive nanocarriers could be applied for tumor treatment and imaging with high spatial resolution simultaneously [63]. And light-sensitive nanoparticles generate singlet oxygen for tumor ablation by photodynamic therapy [64].

Except for the high potential possessed by cancer nanomedicine, we should also consider the practical application in the clinic. Like other scientific advances that have revolutionized medicine over the past decades, cancer nanomedicine also should mature before running into full impact. Overcoming

drawbacks such as reproducibility, scale-up manufacturing, and safety, is key for their translation in the clinic. With more than 200 ongoing clinical trials in last decades, only 14 systemically administrated and 2 locally administrated formulations have been approved globally, which are majorly liposome-based chemotherapy. Unfortunately, no actively targeting or stimuli-responsive formulations are currently on the market. Recently, scientists are also seeking possibilities to shift the nature of the therapeutic agents delivered, e.g. genetic therapy and immunotherapy, besides focusing on the formulation itself. Most of such formulations are still being tested in early stage (phase I or II), but we could foresee there might be some products holding potential to shape the future of clinical practice. Especially, after the tremendous success of lipid-nanoparticle to deliver mRNA (Spikevax® by Moderna and Comirnaty® by Pfizer/BioNTech) to combat COVID-19 and siRNA (Onpattro® by Alnylam) to treat the polyneuropathy in patient with hereditary transthyretin-mediated amyloidosis. The development and scaling up of these lipid-based nanoparticles may also shed light on cancer nanomedicine.



*1.3 Nanomedicine and biological interaction*

To better connect laboratory outcomes with their clinical application, having a good understanding of the intricate interactions between nanoparticles and the biological environment would be beneficial. A full understanding of the complexity of nano-biological interactions helps researchers to dig out a nanoparticle's journey towards the tumor, which benefits future clinical success in cancer nanomedicine.

Nanoparticles used as drug carriers have shown enormous potential to address some biologically caused drawbacks including low internalization and accumulation [33]. Upon systemic administration, these nanoparticles face multi-stage interactions in vivo, including mononuclear phagocyte system, tumor vascular barriers, tumor stroma, the cancer cell membrane and cellular organelles [65]. All these components can dampen the drug accumulation at the target site.

First, when nanoparticles enter the biological environment, their surfaces are rapidly surrounded by proteins and other biomolecules, generating the formation of a ‘corona’ [66]. The protein corona changes the biological identity of nanoparticles and presents a double-edge sword in their travel to the target tissue. It could trigger immunological identification and clearance by preventing binding between targeting ligands with specific receptors and therefore lead to off-target effects. However, on the other side, the protein corona could facilitate the treatment of disease sometimes, for example, Onpattro® utilized ApoE in the protein corona to precisely target hepatocytes and subsequently silence the expression of protein transthyretin. Secondly, the nanomedicine could be primarily trapped by resident macrophages in the spleen and liver before they reach the tumor [67]. For instance, the binding of opsonin (complement protein mediating phagocytosis) with nanoparticles can trigger clearance by the mononuclear phagocyte system and even lead to chronic toxicity [68]. This inspired researchers to modify nanoparticles with PEG to extend their circulation in the bloodstream.

The extravasation from the systemic circulation into tumors can be also affected by aberrant tumor vasculature. The idea that all tumors have a leaky vasculature is being questioned since tumor vasculature is also highly heterogeneous among cancer types and patients [69]. Due to the rapid proliferation of tumors, cells and non-cellular components in the TME generate stress in the tumor tissue, leading to compression of blood vessels [70]. This high interstitial fluid pressure (IFP) within tumor tissue may hinder drug penetration into the tumor stroma and their intracellular infiltration of tumor cells, which is also a major obstacle to effectively deliver nanoparticles [71–73]. In normal tissue, IFP is within the range of 0-3 mm Hg, while solid tumors can reach 5-40 mm Hg, and even 75-130 mm Hg in highly proliferative pancreatic tumors [74]. Increased IFP and decreased microvascular pressure exert fluid stress on the vascular wall, causing the collapse of the tumor vascular system, hindering drug entry into the tumor gap, and resulting in drugs unable to reach deep area [75].

At the same time, tumors with dense stroma hinder a deep and uniform perfusion of nanoparticles, which is due to the aggregation of tumor-related matrix cells, high collagen content, and fiber alignment in the tumor extracellular matrix. Nanoparticles that should have accessed the tumor cells are often depleted by stromal cells [76], or extensively taken up in superficial tumor cells if they actively target to specific receptors, resulting in less opportunity of further penetration [77]. Therefore, targeting tumor cells or tumor-related matrix cells to reduce unsatisfied depletion and increase perfusion can also improve the effective distribution of nanoparticles.



Last but not the least, once the nanoparticles penetrate and accumulate in the tumor tissue, effective cell internalization is necessary to improve therapeutic outcome as many therapeutic cargoes act on intracellular targets. Depending on the biological identification based on the physiochemical properties, cellular internalization of nanoparticles relies on multiple mechanisms such as endocytosis (e.g. clathrin or caveolae-mediated), phagocytosis, micropinocytosis, etc. [78]. Understanding the interaction between nanoparticles and the cell membrane gives insight into the efficient uptake of nanoparticles in living cells. Multiple properties of nanoparticles including size, composition, shape, functionalization, and hydrophobicity, affect the functionality and/or integrity of cellular membranes on their journey inside the cell [79]. For example, morphological changes of nanoparticles alter their interaction with bio-membrane, where small nanoparticles decrease phospholipid lateral mobility to cause a microsized opening and large nanoparticles promote membrane wrapping [80]. Furthermore, once nanoparticles enter the cell, they might be trapped in intracellular components, such as lysosomes, where the cargo could be degraded or excrete in the absence of an adequate escape route.

## 2. Liposomes

As a highly adaptable therapeutic platform, liposomal drug delivery is widely used for the treatment and diagnosis of multiple disease. This system

consists of spherical vesicles composed primarily of lipids and fatty acids, which are inherently biodegradable and biocompatible [81]. Structurally, each phospholipid in the bilayer consists of a hydrophilic head and a hydrophobic tail, in which they naturally assemble in aqueous environments into a lipid bilayer. These bilayers, with hydrophobic tails shielded inside and hydrophilic head towards aqueous environment, form vesicles ranging in size from nanometers to micrometers. Due to the amphiphilic structure, their core can encapsulate water-soluble substances, while the lipid bilayer can encapsulate lipophilic molecules.

Liposomes can contain natural or manufactured lipids, sterols, and surfactants. The backbone of lipids is normally a phosphate, glycerol, or sphingosine group, while the hydrophobic tails can differ in degree of saturation, symmetry, and acyl chain length. The chemical difference of this feature contributes to bilayer assembly via lipid packing, which is connected with particle's physiochemical properties, including stability, encapsulation efficiency, and release profile, etc. For example, lipids with long, saturated acyl chains form liposomes that are more stable in vivo than those with short, unsaturated chains [82]. Natural and synthetic lipids are widely applied to the formation of liposomes. In practice, synthetic lipids are preferred over natural lipids because of their high purity, commercial availability, chemical functionality, and cost-efficiency. Cholesterol is a sterol found in almost all living creatures that is commonly utilized to improve liposome stability and permeability. The incorporation of cholesterol offer liposomes higher stability in the bloodstream for more than 6h, but cholesterol-free liposomes barely lasted a few minutes [83]. Furthermore, to reinforce the stability of liposome in vivo, polyethylene PEG is often used for long circulation purpose, which is highly hydrophilic and thereby reducing opsonization to avoid repaid clearance.

The different techniques for the formation of liposomes have been extensively investigated, including thin-film hydration, reverse-phase evaporation, ethanol injection, freeze-thaw, microfluidics, etc. Among these production methods, thin-film hydration is used most frequently. In this method,

both lipids and lipophilic molecules are dissolved in an organic solvent such as chloroform and then evaporated under vacuum to generate a thin film of lipids. This lipid cake is subsequently hydrated with a predesigned buffer which may contain hydrophilic molecules to obtain roughly generated liposomes. During this process, the temperature of aqueous liquid must be higher than the gel-liquid phase transition temperature of the lipid, and both the volume and hydration rate affect encapsulation efficiency [84]. Following hydration, particle size and their lamellarity can be controlled by sonication and/or extrusion. The size distribution depends on the diameter of membrane pore in extrusion process and the frequency/duration of ultrasonic waves in sonication process.



The unique features of liposomes, such as targeted drug delivery, which leverages specific components to interact with specific cellular receptors, exemplify their potential in precision medicine. Liposomes have been widely demonstrated to improve pharmacokinetic behavior, biodistribution, anti-tumor efficacy, and reduce side effects [85]. Their biocompatibility and ability to encapsulate both hydrophilic and hydrophobic drugs enhance the therapeutic index of treatments by maximizing efficacy while minimizing side effects. However, they also have limitations, including a restricted capacity for drug loading, insufficient stability, and leakage issue in the bloodstream. Hence, while liposomes present an array of clinical benefits over traditional and other nanoparticle-based delivery systems, ongoing research and developments are crucial to overcome their limitations and fully exploit their capabilities in medical science.

### 3. Liposome application in cancer therapy

Since the development in the 1960s by Alec Bangham [86], liposomes have evolved considerably from a laboratory curiosity to essential component in clinical applications, especially in targeted drug delivery for cancer therapy and vaccines. Several liposomal formulations were approved by either U.S. Food and Drug Administration or the European Medicine Agency for clinical use [87]. A wide range of chemotherapeutics can be loaded into liposomes for

various cancer treatments, including but not limited to camptothecin, cisplatin, daunorubicin, docetaxel, doxorubicin, gemcitabine, oxaliplatin, paclitaxel, and vincristine etc. In practice, liposomes can be either used directly to eliminate tumor cells or to modulate immune response, depending on their payload and inherent features.

*3.1 Liposome application in cancer therapy to directly inhibit tumor growth*

In cancer therapy, liposomes have demonstrated to be particularly useful due to their ability to reduce side effects while enhancing anti-cancer efficacy. Generally, chemotherapeutic agents are highly toxic to both cancer and normal tissues, representing the main challenge in their application. However, the incorporation of chemotherapeutics into liposomes improves their selectivity to cancer cells, and consequently reducing side effects. Meanwhile, increased drug accumulation within tumors facilitates the anti-cancer efficacy. Moreover, many chemotherapeutics need to maintain a certain concentration to be effective, but they are cleared rapidly and have low bioavailability. PEGylation of liposome can reduce drug clearance by immune and renal systems, thereby extending the circulation time of anti-cancer drugs and increasing their availability at the tumor site.

As the first FDA approved liposomal formulation, DOXIL® encapsulates doxorubicin HCl and is composed of L-α-phosphatidylcholine hydrogenated soy, cholesterol and N-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt in a molar ratio of 56:38:5. Compared to the free doxorubicin, DOXIL® displays an increased circulation time with a half-life of 20-35 h and significantly lower cardiotoxicity [88]. Onivyde™, a liposomal formulation of irinotecan comprises distearoylphosphatidylcholine, cholesterol, and pegylated 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine in a molar ratio of 3:2:0.015, received FDA approval in 2015 for the second-line treatment of pancreatic ductal adenocarcinoma. In human colon (HT29) and breast (BT474) cancer xenograft models, the liposomal formulation has an improved drug loading and longer

half-life in comparison to free irinotecan, resulting in a significant increase of cytotoxicity [87]. In addition to the clinical formulations, a large number of liposomal delivery systems for cancer therapy are being developed in laboratories enter the preclinical stage.

Appealing evidence indicate that liposomes are also very suited for the delivery of multiple compounds simultaneously, also known as co-delivery. Liposome-based co-delivery increase the drug antiproliferative activity, broaden the application field, and elevate the potency of combinational drug regimen, while still decreasing systemic toxicity. Principally, liposomes enable synchronization and control of the pharmacokinetics and biodistribution of the drugs, along with uniform time and spatial co-delivery of two regiments. For example, it has been reported that liposomes co-loaded with salinomycin and doxorubicin exhibited sustained release of both drugs. Both in vitro and in vivo results revealed more effective tumor eradication than monotherapy [89]. Furthermore, it has been shown that a rational combination of anti-cancer drugs and other agent types can sensitize cancer cells and overcome drug resistance, such as with gene therapy. For example, siRNA targeting of multidrug resistance mutation (MDR1) gene can decrease the formation of efflux transporters at the cell membrane, resulting in an increase in cellular drug concentration [90]. This opens a window in which the chemo-resistant cells transiently become sensitized to the anti-tumor drug, thereby overcoming multi-drug resistance. Long et al. designed PEGylated liposomes for the co-delivery of Bcl-2 siRNA and docetaxel to improve the chemotherapeutic efficacy in multi-drug resistant cancer cell lines. These liposomes demonstrated a prolonged blood circulation of docetaxel and the synergistic effect of docetaxel and resistance-reversing siRNA led to 100% survival rate of established lung cancer model [91]. While the combination of multiple therapeutic regiments based on liposomal system offers new therapeutic potential, it simultaneously exposes several challenges. These aspects are discussed in greater detail in **chapter 2**.

*3.2 Liposomes in cancer therapy to regulate immune system*

The promising potential of liposomes is highly associated with their interaction with the immune system. Physiochemical properties including size, lipid composition, charge, surface characteristics, bilayer packing, as well as practical factors such as dose and injection method, can all contribute to a robust immune response [92]. There are many studies emphasizing how size affects dendritic cell (DC) internalization and subsequent T cell priming [93,94]. For example, liposomes larger than 200nm are preferentially taken up by micropinocytosis, whereas smaller liposomes tend to be internalized by endocytosis [95]. It has been reported that small liposomes (<100nm) following phagocytosis and endosome route gain a higher opportunity to promote T cell priming [96], and display robust interferon (IFN)-γ release leading to Th1 immunity in mice [97]. Besides, various lipids can be assembled into liposomes within a proper ratio, and their electrical charge can also influence how liposomes interact with target cells. For example, cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) have been widely reported their vaccine-elicited anti-tumor immunity when served as adjuvant alone [98]. This effect is primarily attributed to the interaction between positive charged lipid and negatively charged cell membrane, and their effects on the upregulation of co-stimulatory molecules (e.g., CD80 & CD86) and IFN-γ [99].[102] Apart from size and charge, rigidity can also affect the immune response as well as biodistribution, as liposomes with higher rigidity are easier accessible by DCs than those less rigid [94,100,101]. In line with this theory, a study has confirmed that injection of more rigid DSPG-liposomes (DSPG, 1,2-distearoyl-sn-glycero-3-phosphoglycerol) can introduce stronger Treg responses [102]. Similarly, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC-PEG) liposomes with more solid gel-phase show higher internalization in bone marrow-derived DCs and stimulate cells more than liposomes with fluid-phase [103]. Moreover, dose and the route of administration should also be considered carefully as they are crucial for both side effects and therapeutical effect. For example, cationic liposomes can bind to hemoglobin and lead to hemolysis, which makes it unsuitable for systemic administration. Except for

the inherent properties to influence immune responses, there are more parameters and factors of liposome-based immunotherapy to modulate anti-cancer immunity, which is extensively discussed in **chapter 2**.

To generate a potent immune response, several considerations should be taken into account when designing a liposomal formulation. A common approach to improve the therapeutic index of drugs is to combine different cancer therapies with liposome-based technology, partially or entirely. We summarized three common strategies in the application of liposomal-based drug delivery systems for stimulating immune response (Fig. 2): (1) In situ vaccination: employing the potential of liposomes for the coordinated delivery of cytotoxic drugs and other stimulatory molecules to the TME, which may stimulate induced immunogenic cell death (ICD), a process of immunogenic apoptosis that can amplify certain types of immune responses. (2) Normalization: killing cancer cells directly while overcoming tumor-driven immunosuppressive signals in the TME, especially on cancer cells and T cells. A typical example is the combination between various chemotherapeutic agents with immune checkpoint blockade (ICB) therapy. (3) Modulation: regulating existing or known pathways during the development of the anti-tumor response. In addition to tumor cells and T cells that contribute to a suppressive milieu, there are other cells and pathways with the ability to inhibit anti-tumor immunity. Targeting these cells/pathways can also manipulate the development and progression of a tumor, thereby creating a favorable environment for infiltration of effector cells within the tumor.

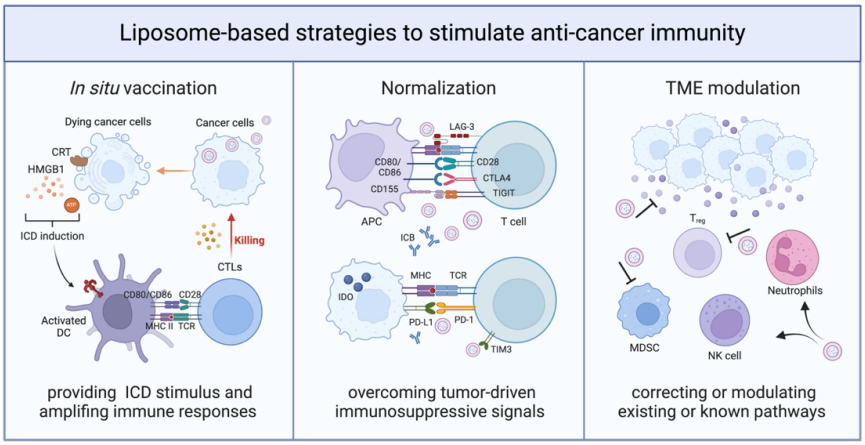


**Fig. 2. Different strategies of liposomes employed in the treatment of cancer therapy to stimulate immune responses.** 1) *In situ* vaccination: Liposomes carried with specific chemotherapeutical agents can convert cancer cells into in situ 'vaccine' by inducing ICD. This process can be assisted by the damage-associated molecular patterns (calreticulin (CRT), high-mobility group box 1 protein (HMGB1), ATP, etc.) released from apoptotic cancer cells, which stimulates antigen-presenting cells (e.g., DCs) and subsequently trigger T cell priming. Activated cytotoxic T cells (CTLs) then induces tumor cell lysis or apoptosis via secreting effector molecules such as granzyme, IFN, and tumor necrosis factor, etc. 2) Normalization: Immune escape processed by cancer cells and T cell itself can inhibit T cell activity in the tumor microenvironment to prevent immune attack. This is mediated by specific molecules expressed on them, such as immune checkpoint receptors. Targeting these receptors by specific liposomal therapy or blocking antibodies can reverse the immunosuppressive state and T cells may recover the cancer cell killing ability. 3) Modulation: Innate immunity has a considerable impact on how the adaptive immune response is activated. Multiple immune cells (myeloid-derived suppressor cells (MDSC), natural killer (NK) cell, neutrophils, etc.) and some of their

pathways can be manipulated to regulate anti-tumor immunity and generate robust inflammatory responses. (Created with BioRender.com)

*3.3. Liposomes in cancer therapy for synergistic/additive immune-regulation*

In tumor tissue, tumor cells interact with stromal cells and form a unique microenvironment. These stromal cells provide cancer cells a “protection shield” to external interventions, thus allowing them to remain dormant for a long time. It is a complex system composed of many components, including endothelial cells and their precursors, smooth muscle cells, various phenotypic fibroblasts, myofibroblasts, neutrophils, and other granulocytes (eosinophils and basophils), mast cells, etc. Due to the complexity of tumors and their microenvironments, a single drug or treatment strategy may be insufficient for effective tumor therapy, making combination therapy the main choice for good therapeutical effect and prognosis. It involves the simultaneous use of multiple therapeutic agents or approaches to target different aspects of cancer biology, often at various stages of tumor development. These approaches include chemotherapy, immunotherapy, targeted therapy, and radiation therapy, among others. Normally it improves anti-tumor efficiency through multiple ways such as additive effects, synergistic interactions, or potentiation [104–107]. Among various choices, the combination between immunotherapy and other regiments appears to be attractive in the last decades.

The combination of immunotherapy with traditional anti-cancer drugs (chemotherapy, targeted therapy, etc.) provides synergistic or additive effects to improve therapeutic efficiency of cancer. Chemotherapy normally kills cancer cells directly while immunotherapy stimulates immune response to deplete cancer cells. Given the rapid but relative short action time of chemotherapy, the effects of immunotherapy plus chemotherapy are complementary since immunotherapy induces potent responses and long-term immune memory. Besides, chemotherapy has been showing great potential in many preclinical studies that they could offer additional benefits to immunotherapy, for example,

triggering immune responses by multiple mechanisms [108]. Understanding the immunomodulatory effects of conventional chemotherapy can improve the development of novel, effective treatment options in which such molecules are combined with immunotherapies. Here we briefly introduce some types of chemotherapeutical agents included in this thesis, which have been reported to have immunomodulation effects.

Taxanes are well-known to inhibit tumor growth by directly binding to microtubules and interfering with the mitotic spindle functions. Research has shown that part of the therapeutic effects can be attributed to cancer cell-extrinsic immune mechanisms [108]. It has been reported that paclitaxel could impair the function of regulatory T cells [109] and make cancer cells more accessible to cytotoxic T lymphocytes by increasing their permeability to granzyme B (utilized by T cell or natural killer cells to kill cancer cells) [110]. Another taxane called docetaxel was also reported to have immunostimulatory effects. It was initially used for the broad-spectrum treatment of multiple cancers, but shown potential to increase calreticulin (CRT) expression and deplete MDSCs in mice, leading to enhanced anti-cancer responses [111].

Folate antagonists were developed to inhibit the production of tetrahydrofolate and therefore to inhibit the synthesis of nucleotides in tumor cells, leading to cell apoptosis [112]. In light of Sidney Farber's success [113], with anti-folates as the first mechanism-based metabolic therapy for cancer, metabolic drugs have been receiving continuous attention in both single and combinational sue. Methotrexate targets dihydrofolate reductase and has played an important role in the development of cancer chemotherapy. Pemetrexed appeared later and targets thymidylate synthetase and 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, adding to the therapy regimens against non-small cell lung cancer primarily. Recent studies suggest that low dose of the second-generation folate antagonist pemetrexed could boost DC activation and exert T-cell intrinsic effects by augmenting mitochondrial function and enhancing T-cell activation in vitro. Furthermore, when combined with programmed death-ligand 1 (PD-L1) blockade,

pemetrexed displayed improved anti-tumor effects and promoted immune activation since it upregulates PD-L1 expression and primes a favorable microenvironment for immune checkpoint blockade [114].

Preclinical data reveals that anthracyclines, such as doxorubicin and daunorubicin, rely on immune mechanisms to exert anti-tumor effects. Doxorubicin has been demonstrated to induce robust ICD and further enhance the proliferation and infiltration of tumor antigen-specific CD8+ T cells [115]. Similar to anthracyclines, the third-generation platinum-based drug oxaliplatin, which is frequently used for colorectal cancer through DNA damage, can induce ICD and promote T cell priming, too. Oxaliplatin stimulates pathways involved in (1) the translocation of CRT from the endoplasmic reticulum to the cell surface and (2) the release of high-mobility group box 1 protein (HMGB1), of which both are important immunogenic signals for the initiation of DC maturation [116]. Additionally, oxaliplatin is also reported to activate the stimulator of interferon genes (STING) pathway through damage of DNA (which triggers the STING pathway) and to increase the percentage of M2 macrophages (anti-inflammatory) in the tumor microenvironment, which offers more targets for the combination of oxaliplatin with other regiments.

Targeted therapeutic drugs have emerged as a viable strategy for cancer treatment due to their superior efficacy and safety compared to standard chemotherapy drugs. Targeted drugs can modulate specific pathways overexpressed in cancer cells, hence having high potency and may induce lower toxicity in healthy cells that do not overexpress the target pathway. Protein kinase inhibitors (TKI) are one of the most interesting due to their good pharmacokinetic performance and patient compliance. Most tyrosine kinase receptor inhibitors are designed to target the epidermal growth factor receptor (EGFR), platelet-derived growth factors receptor and vascular endothelial growth factor receptor tyrosine kinase families [117]. Recently, EGFR inhibition has been linked to increased expression of MHC I and II molecules, potentially boosting cancer-directed immunological and/or inflammatory responses. For example, sunitinib and sorafenib have been linked to decreased infiltration of

regulatory T cells and MDSC in patients with colorectal cancer [118,119]. Along similar lines, cabozantinib, a multi-target TKI inhibitor (EGFR, c-MET, AKT), induces tumor clearance in mice by also increasing both intratumor infiltration and killing ability of neutrophils and T cells, through upregulation of chemokines (e.g., CCL11, CCL8, etc.) or HMGB1 neutralization [120,121]. These targeted anti-cancer drugs often influence the immune system and may subsequently induce anti-cancer immune responses.

Overall, conventional anti-cancer drugs can activate the immune system against cancer cells by at least the following ways: 1) directly activating effector T cells, leading to the production of cytokines and chemokines (e.g. interleukin (IL)-2, IFN-γ, etc.); 2) stimulating natural killer cell and DC functions; 3) inhibiting or depleting immunosuppressive MDSCs and regulatory T cells; 4) inhibiting other immunosuppressive molecules/pathway, such as PD-1/PD-L1 axis and indoleamine 2,3-dioxygenase (IDO); 5) triggering ICD; upregulating MHC-I molecules on cancer cells.

To summarize, combining multiple regimes is likely to intensify cancer toxicity and overcoming immune escape, which may be challenging to manage. Achieving a balance between efficiency with side effects requires extensive testing and adjustment. For instance, simply injecting different components to patients usually lacks selectivity, presents fast clearance, and is unable to cross biological barriers (blood-brain-barrier, tumor microenvironment, cell membrane etc.). From this perspective, combination of approaches based on liposomes can provide a well-defined platform to treat patients. Developing this kind of delivery systems that integrate various therapeutic modalities provides a promising approach for enhanced precision and effectiveness in treating different cancer types. The employment of liposomes on these combinational approaches is extensively discussed in **chapter 2**.

## 4. The scope of this thesis

The work presented in this thesis was the result of multi-disciplinary cooperation between the fields of Nanomedicine, Pharmaceutics, Oncology,

and Immunology. The aim was to design and evaluate promising drug delivery systems and drug combinations with the aim to improve cancer therapeutic efficacy via modulating immune responses.

In **chapter 2**, we summarized the potential use of liposomes in the cancer immunotherapy field. In this review, we explain how liposomes can be employed for the regulation of the immune system, alone and together with other regiments. The unique features of liposomes offer solutions to common issues that can occur in cancer immunotherapy such as low response rate and off-target effects. As a delivery system, liposomes high biocompatibility, efficiency as well as adjuvant activity, makes it likely to play an important role in future cancer immunotherapies.



In **chapter 3**, we developed a pH-sensitive liposomal formulation to encapsulate two chemotherapeutics with different physiochemical properties. In this study, we analyzed if liposome-mediated chemoimmunotherapy could improve the tumor-killing ability of immune T cells by chemo-sensitizing cancer cells towards pemetrexed with docetaxel. We aimed to induce strong ICD while also inhibiting cancer immune resistance with anti-PD-L1 co-therapy. We showed that our pH-sensitive liposomal platform has great clinical potential and offers a well-controlled release vehicle by combining chemotherapy with diverse immunotherapies. In **chapter 4**, a clinically standardized liposome system was employed to simultaneously deliver a TKI inhibitor and an IDO inhibitor, thereby achieving improved efficacy, high biocompatibility and safety. We demonstrate that these liposomes can passively target tumor tissues after long circulation period and induce cell death directly by interfering with the metabolism of tumor cells. Moreover, they can also generate a more permissive tumor microenvironment by strengthening anti-tumor immunity.

In the previous two chapters, we focused on inducing potent anti-tumor effects by boosting the adaptive branch of the immune system. With the bridging role on innate immunity and adaptive immunity to achieve potent immune responses, the cyclic GMP-AMP synthase (cGAS) - stimulator of interferon genes (STING) pathway was investigated in our combinational

strategy. In **chapter 5**, we developed liposomes encapsulating oxaliplatin and combined this formulation with a STING agonist for the treatment of a colorectal cancer model. Oxaliplatin-loaded liposomes induced the release of tumor-associated antigens and doubled the DNA release which synergistically promoted STING activation and further immune responses. The liposome-based immunochemotherapy resulted in complete remission by regulating the immunosuppressive state in the tumor and represents a promising strategy for cancer therapy. In **chapter 6**, we designed a double liposomal system to encapsulate a toll-like receptor (TLR) agonist and chemotherapeutics. We have shown that the immunostimulatory liposomes with TLR agonist amplifies the anti-tumor effects induced by liposomes with chemotherapeutics, and potentially regulated the tumor microenvironment especially by inducing a shift of tumor associated macrophages towards an inflammatory subtype. The results from the zebra fish and mice models indicated a promising strategy for efficient treatment of cancer.

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