Combinations of chemo-, immuno-, and gene therapies using nanocarriers as a multifunctional drug platform

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ABSTRACT

Introduction:

Cancer immunotherapies have created a new generation of therapeutics to employ the immune system to attack cancer cells. However, these therapies are typically based on biologics that are non-specific and often exhibit poor tumor penetration and dose-limiting toxicities. Nanocarriers allow the opportunity to overcome these barriers as they have the capabilities to direct immunomodulating drugs to tumor sites via passive and active targeting, decreasing potential adverse effects from non-specific targeting. In addition, nanocarriers can be multifunctionalized to deliver multiple cancer therapeutics in a single drug platform, offering synergistic potential from co-delivery approaches.

Areas covered:

This review focuses on the delivery of cancer therapeutics using emerging nanocarriers to achieve synergistic results via co-delivery of immune-modulating components (i.e. chemotherapeutics, monoclonal antibodies, and genes).

Expert opinion:

Nanocarrier-mediated delivery of combinatorial immunotherapy creates the opportunity to fine-tune drug release while achieving superior tumor targeting and tumor cell death, compared to free drug counterparts. As these nanoplatforms are constantly improved upon, combinatorial immunotherapy will afford the greatest benefit to treat an array of tumor types while inhibiting cancer evasion pathways.

KEYWORDS:

Cancer, chemoimmunotherapy, delivery, gene, immunotherapy, nanomedicine, nanoparticle, nanocarrier

ARTICLE HIGHLIGHTS:

- Cancer immunotherapies are being used to combat the limitations of conventional cancer treatments, including small molecule therapeutics, by modulating the immune system to fight cancer.
- The combination of immunotherapeutics, specifically those targeting HER2 and PD-1/PD-L1, with nanocarriers encapsulating chemotherapeutics has synergistic effects and demonstrates enhanced efficacy.
- Immunotherapeutic ligands can be co-delivered with genes to provide synergy in tumor inhibition by attacking multiple hallmarks of cancer.
- Nanocarriers can be multifunctionalized to deliver genes and small molecule drugs ligands to inhibit multidrug resistance or dually attack pro-tumorigenic pathways
- The development of personalized medicine enabled by multifunctional nanocarriers will allow for the creation of cancer therapy cocktails that elicit the highest impact for a wide range of patients.

1.0 Introduction

The development of cancer immunotherapy created a new perspective on how to effectively treat malignant tumors. Conventional cancer therapeutics have largely involved the discovery and development of small molecule drugs which destabilize cancer cells by disrupting proliferative pathways[1]. However, a major pitfall to small molecule drugs is the intrinsic and acquired resistance mechanisms cancer cells often develop. Cancer cells are continuously adapting to external stimuli by mutating their genomic profiles, inducing the expression of drug transporters, amongst others[2]. To overcome the limitations of small molecule drugs in anti-cancer therapy, cancer immunotherapies can be used, aiming aim to restore immune surveillance[3]. This concept prevents the development of malignant cells as immune cells recognize abnormal cells and facilitate their eradication[4]. For example, the "self" checkpoint interactions involve communication with T cells expressing programmed cell death receptor 1 (PD-1) binding to its ligand, programmed death-ligand 1 (PD-L1)[5]. Additional co-inhibitory binding pairs include cytotoxic T lymphocyte-associated protein 4 (CTLA-4) with CD80/CD86.

Currently, there are multiple FDA-approved immunotherapies that provide blockade of these co-inhibitory binding pairs (**Table 1**). However, similarly to treatment with small molecules, cancer cells can alter their gene expression to overexpress alternative immunosuppressive ligands, achieving adaptive resistance[6]. For example, after anti-PD-L1 treatment, cancer cells can redirect gene expression to overexpress an alternate inhibitory ligand, galectin 9 (GAL9)[6]. GAL9 binds to T cell immunoglobulin and mucin domain 3 (TIM3) expressed on T cells, suppressing anti-tumor immunity[7]. Another downfall to antibody-based immunotherapies is that only 10-30% of patients typically respond to this treatment[6]. Patients with "hot" tumors, or tumors overexpressing an immunosuppressive ligand target with T cell infiltration are optimal candidates[8]. Another reason for poor response outcomes is tumors with innate primary resistance to immunotherapies. Certain types and numbers of immune cells other than cancer cells within the tumor microenvironment (TME) also play a role in determining the primary resistance. Regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and M2 macrophages function to downregulate the immune system. Tregs work to suppress effector T cells (Teffs) through the secretion of inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor

beta (TGF-beta)[9]. In addition, solid tumors with poor T cell infiltration ("cold tumors") do not benefit from immune checkpoint blockade as activated T cell populations can not effectively migrate into the dense extracellular matrix (ECM)[10]. Acquired and primary resistance mechanisms to chemotherapy and immunotherapy stimulate the need for optimized therapies which bypass cancer cell evasion.

Table 1. FDA-approved monoclonal antibody-based immunotherapies[11]

Mechanism	FDA-approved therapies
Anti-PD-1	Nivolumab Cemiplimab Pembrolizumab
Anti-PD-L1	Atezolizumab Avelumab Durvalumab
Anti-CTLA-4	Ipilimumab
Anti-LAG-3	Relatlimab [12]

2.0 Combination Therapies

The combination of multiple therapies as one treatment regime is termed combination therapy (**Figure 1**). Combination therapies allow for the ability to maintain manageable safety profiles as dosing regimens can be lowered while maintaining efficacy due to synergistic outcomes[13]. For the treatment of advanced melanoma, the combination of dual immunotherapies such as anti-PD-1 and anti-CTLA-4 monoclonal antibody drugs (mAb) at doses of 1 mg/kg and 3 mg/kg, respectively, showcased tumor regression of 80% or more compared to <3% of patients on monotherapy at a 3 mg/kg dose[14]. Combinatorial chemoimmunotherapy involves the co-delivery of traditional chemotherapeutics with immune checkpoint inhibitors in order to dually target multiple hallmarks of cancer, disseminating opportunities of adaptive resistance against cancer. Traditional chemotherapeutics aim to induce cell death and/or inhibit proliferative signaling, whereas immunotherapies prevent cancer cells from the evasion of immune destruction[15]. The combination of these therapies is particularly beneficial as cytotoxic drugs provide immediate cancer-killing effects and immunotherapies offer sustained efficacy after treatment is finished. Certain chemotherapies, including anthracyclines, cyclophosphamide, and oxaliplatin, have an immunomodulating feature as they induce the release of tumor-associated antigens (TAAs)[16,17]. Dendritic cells can capture the released TAAs and present these antigens

through major histocompatibility class (MHC) molecules to T-cells. This interaction causes CD8+ T cells to recognize the abnormal proteins expressed by cancer cells, causing their subsequent destruction[16,18]. This cascade falls under passive immunotherapy, a transient immune-activating effect only lasting the duration of treatment. Other passive immunotherapy approaches include targeted monoclonal antibodies. Anti-HER2 are monoclonal antibodies targeting the TAA, human epidermal growth factor receptor type 2 (HER2), frequently overexpressed on cancer cells. When HER2 is blocked, oncogenic intracellular pathways are downregulated, and the Fc-gamma-receptor on natural killer (NK) cells can recognize the crystalline fragment (Fc) domain of the mAb. This interaction causes antibody-dependent cellular cytotoxicity[19].

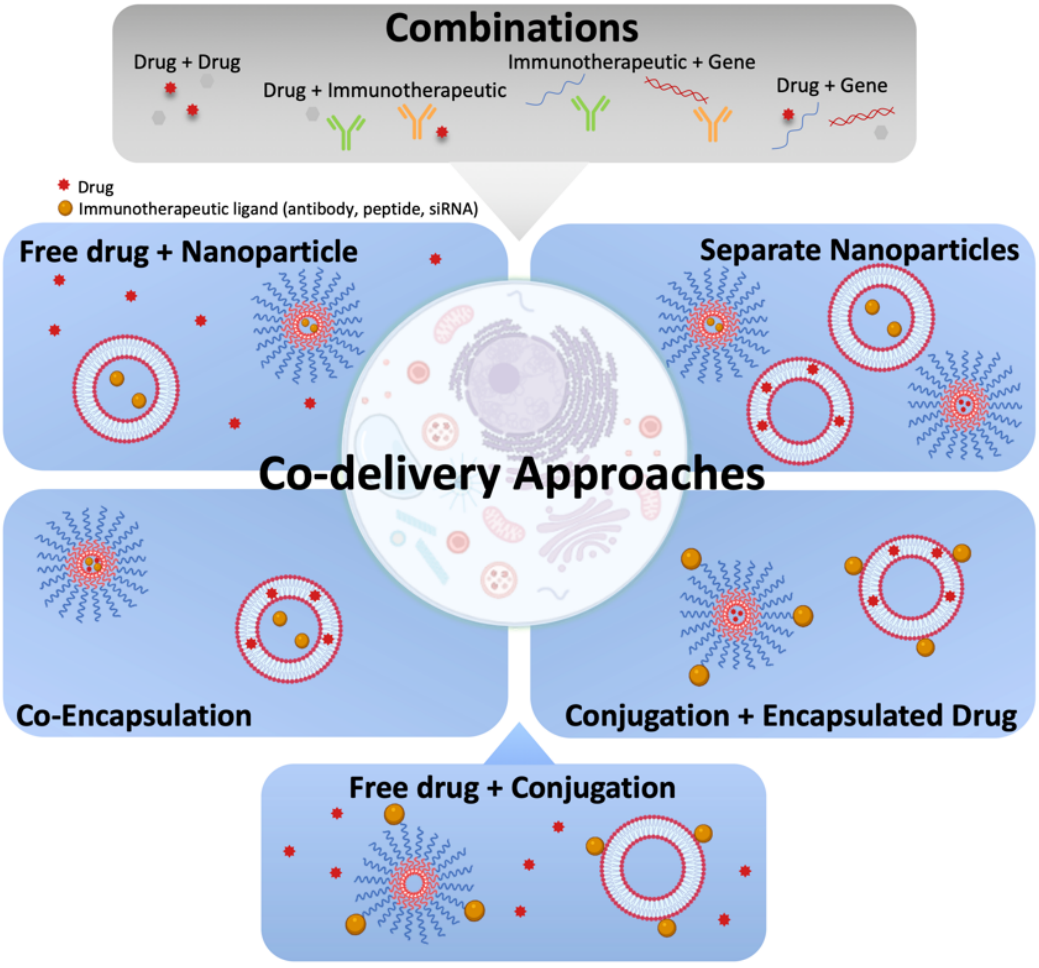

Figure 1. Nanocarrier-mediated combination approaches for cancer therapy.

With the development of combination therapies, a range of treatment options can be combined to provide synergistic outcomes in tumor regression. Gene therapy, for example, gives the opportunity to directly modify the tumor cells or indirectly alter the tumor microenvironment[11]. The goal of delivering DNA plasmids, messenger RNA (mRNA), small interfering RNAs (siRNA), or microRNAs (miRNAs) are to upregulate anti-tumorigenic pathways and downregulate pro-tumorigenic ones. However, achieving successful gene therapeutic outcomes for cancer have been proven difficult as there are only three FDA-approved products[11]. Systemic administration of genetic material alone leads to degradation as a result of nucleases in the blood[20]. Cellular internalization of genetic material is also limited due to the anionic charges from the phosphate backbone limiting cellular uptake across anionic plasma membranes[21]. Consequently, viral vectors, such as adenoviruses, have been developed as effective gene carriers with high transfection activity[22]. Current FDA-approved gene therapies are viral-mediated, running the possibility of an immunogenic response, potential transgene mis-insertions, and result in high manufacturing costs[23]. As a result, non-viral gene vectors have attracted tremendous research interest. Nanocarriers have the advantage of preferentially accumulating in the tumor due to the enhanced permeability and retention (EPR) effect[24]. In addition, the nanocarrier surface can be functionalized with targeting moieties to allow for active targeting of cargos to sites of interest (**Figure 2**)[8,25-28]. More specifically, active targeting often involves the use of a cancer-specific antibody ligand which can augment specificity from the EPR-effect-based passive targeting of nanocarriers[29]. Other methods to increase therapeutic efficacy of cancer nanomedicines include the use of photothermal therapy[30] and hyperthermia[31], amongst others[32]. To achieve a successful combination of chemotherapy or immunotherapy with gene therapy, a gene vector is required. Non-viral gene vectors, such as lipid-based cationic or ionizable carriers, can condense genes, providing protection from nucleases and allowing efficient transport across cell membranes and endosomal escape[21]. Essentially, non-viral nanocarriers have the potential to have co-delivering capabilities in order to deliver cytotoxic drugs, immune checkpoint inhibitors, and genes within a single platform while maintaining biocompatibility.

Another important limiting factor in co-delivery approaches is different pharmacokinetic profiles for mAbs, small molecule drugs, and genetic materials. Currently, all mentioned treatment options are systemically administered, non-specifically distributed to both target and non-target cells[13].

This issue is particularly seen in patients treated with chemoimmunotherapies, as studies found patients showed grade 3 (moderate-to-severe adverse effects) or higher adverse events (life-threatening symptoms), forcing 10-20% of patients to discontinue treatment as a result[33,34]. Therefore, to optimize the therapeutic benefit from a combination approach, all cancer therapies must be tuned to specifically target tumor cells, decreasing the risk of adverse events. This review will thus highlight the different nano-scale drug carriers used to improve combinatorial cancer immunotherapies.

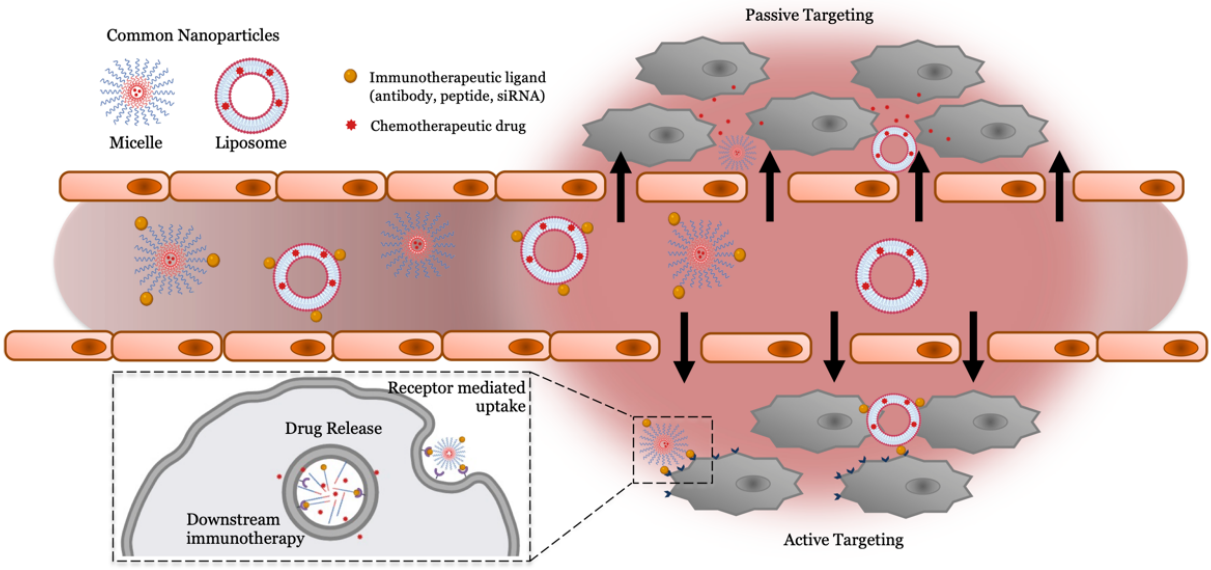

Figure 2. Nanocarriers for active and passive chemoimmunotherapy.

3.0 Chemotherapy and immunotherapy combination

One of the most common combinatorial cancer approaches is the combined use of an immunotherapeutic targeting ligand, such as an antibody, and a chemotherapeutic drug. The immunotherapeutic allows for immunostimulation, combined with the chemotherapeutic causing cytotoxicity of the cancerous cells. Nanocarriers for this delivery allow for reduced off-target effects, more efficient delivery, and enhanced tumor penetration (**Table 2**).

3.1 Targeting HER2 with passive immunotherapy

Antibodies are commonly employed targeting ligands for various therapeutics, not only for their efficient binding with upregulated receptors on cancerous tissues, but for their immunotherapeutic downstream effects as well. As one of multiple types of immunotherapies, antibodies facilitate passive immunotherapy, where an antibody targeting a tumor antigen can subsequently cause anti-tumor effects without the need of a patients innate immune system[35,36]. While active immunotherapy causes a longer-term response by inducing immune memory, passive immunotherapy results in immediate response via antibody- or cytokine-receptor interactions. For instance, HER2 is a transmembrane tyrosine kinase receptor that is overexpressed in approximately 25-30% of breast cancers, serving as a popular target[37,38]. Overexpression of HER2 has been related to poor prognoses, specifically seen in decreased overall survival and decreased sensitivity to chemotherapeutics[38]. Targeting the extracellular domains of HER2, Trastuzumab has been a prevalently used monoclonal antibody since its FDA approval in 1998[39,40]. Upon binding with the receptor, the antibody blocks the downstream effects that signal for survival, proliferation, and invasion, specifically through causing G1 cell cycle arrest and inhibition of PI3K/Akt pathways, blocking angiogenesis and inducing apoptosis[40-42]. When bound with HER-2 Trastuzumab also causes antibody-dependent cellular cytotoxicity by interacting with NK cells and marking the cancer cells for immune attack[41,43,44]. To provide synergistic effects, antibody-drug conjugates (ADCs) have been developed, but suffer from fast clearance, off-target effects, immunogenicity, and poor tumor penetration[45]. Conjugation to nanocarriers addresses the issues by allowing for increased blood circulation, passive targeting through the EPR effect, and biocompatibility, to name a few advantages. In turn, antibody-conjugated nanocarriers can also be combined with chemotherapeutic drugs for more effective delivery and combinatorial effects.

Bolu et al. developed docetaxel-loaded micelles composed of dendron-polymer conjugates functionalized with trastuzumab[46]. The targeted micelles demonstrated increased cellular internalization and delivery of cargo compared with non-targeted micelles, including continuous uptake over time. This result is influenced by the dendron polymer offering multivalent binding of the HER-2 targeting arm, inducing receptor mediated endocytosis, thus enhancing localization of therapeutic cargo inside the cell[47,48]. In order for the encapsulated drug to become bioavailable, degradation of the dendron based micelle must occur. Due to the dendron being composed of

polyester groups, hydrolysis of ester bonds occurs inside the acidic endosomes to release cytotoxic payload in a localized manner. Enhanced efficacy of drug/immunotherapeutic combinations was also observed when comparing cytotoxicity of drug-loaded non-targeted nanocarriers, targeted nanocarriers, and free drugs. Trastuzumab-conjugated micelles demonstrated significantly lower cell viabilities and EC_{50} values in two different cell lines[46]. Strategies for combining Trastuzumab with a nanocarrier vary, ranging from conjugation to complexation. Using a different drug, Lee et al. also demonstrated the combinatorial effects of chemotherapeutics and trastuzumab targeting and passive immunotherapy[49]. The combination of trastuzumab (referred to by the commercial name Herceptin® in their paper) and paclitaxel was accomplished via antibody complexation with cationic micellar nanocarriers. The cytotoxic effects of paclitaxel were increased when co-delivered with trastuzumab.

When developing a delivery system for treatment, surface charge plays a role in toxicity and cellular interactions, with a positive surface causing toxicity within the body. Lee et al. reported a micelle of zeta-potential of 60 mV, which raises concerns of off-target toxicity due to the positive charge[49]. The complex micelle was generated via electrostatic interactions, which required a cationic nanocarrier, but this toxic positive charge could be mitigated by subsequent deactivation of remaining positive charges or covalent conjugation, resulting in neutral or slightly anionic nanocarriers. Zhou et al., on the other hand, covalently conjugated trastuzumab to their poly(D,L lactide-co-glycolide) (PLGA)-b-poly(L-histidine) (PHis)-b-polyethylene glycol (PEG) tri-block copolymer nanocarriers via a click reaction[50]. The PHis proton sponge polymer was utilized to enhance pH sensitivity and endosomal escape of encapsulated doxorubicin. When compared with non-targeted nanocarriers, trastuzumab-conjugated nanocarriers demonstrated increased cellular uptake and the greater therapeutic efficacy. Not only does conjugation of Trastuzumab to the surface of a nanocarrier increase the targeting ability, the antibody itself also has better tumor delivery potential via the EPR effect afforded by the size of the conjugated nanocarrier.

Using a HER2-targeted, doxorubicin-loaded liposome (MM-302) already in clinical trials, Espelin et al. aimed to go one step further and achieved dual binding of HER2 via trastuzumab on domain IV and MM-302 on domain I of the target protein[51]. MM-302 is a doxorubicin-loaded liposome with single-chain anti-HER2 antibodies (scFv) on the surface that is currently undergoing clinical trials for HER2-positive breast cancer treatment. The single-chain antibodies on MM-302, which cause DNA damage, bind to a different region of the HER2 extracellular domain than trastuzumab,

allowing for concurrent binding and downstream effects. Not only did they find an increase in cellular interactions and uptake of MM-302 when combined with trastuzumab, synergistic anti-cancer effects were also observed in the form of reduced tumor volume and tumor regression. In comparison to micellar forms, liposomes offer the ability to encapsulate both hydrophobic and hydrophilic drugs without the need to chemically modify the nanocarrier. In contrast, micelles must be chemically engineered to encapsulate either hydrophobic or hydrophilic drug[52,53]. In addition, liposomes offer enhanced stability compared to micelles that can dissociate once concentration is below the critical micelle concentration (CMC) post-injection into the body[54]. A recent study has found higher levels of tumor-infiltrating lymphocytes (TILs) and PD-L1 expression in HER2-positive breast cancers, placing greater emphasis on further combination efforts[41]. Above has already seen the combination of HER2-targeted passive immunotherapy with chemotherapeutic drugs, but the addition of PD-L1 immune checkpoint blockade can provide additional synergistic effect, further bolstering the immune system to fight the cancerous cells.

In order to continuously augment tumor regression, treatment regimens may benefit from alternating or combining therapies targeting one tumor antigen over another. More studies elucidating the priority of different tumor antigens over a variety of cancers can offer insight into optimal treatment plans. In addition, questions whether initial injection of combination therapies offer greater therapeutic effects over a gradual titration of one therapy to another must be validated in order to optimize treatment plans. This perspective on therapy regimes could potentially aide in the prevention of drug resistance and thus maintain tumor regression.

3.2 Targeting PD-1/PD-L1 checkpoint blockade

An active immunotherapeutic commonly uses a receptor pair for immune checkpoint blockade and can cause a downstream immunotherapeutic response in the PD-1/PD-L1 pathway, which is frequently found in tumors[55]. The PD-1 receptor is expressed on regulatory T cells (Treg) and TILs, whereas PD-L1 is upregulated on tumor cells. Binding between the two receptors results in immunosuppression, so blockade of this interaction allows for activation of the immune system and subsequent recognition of tumor cells, leading to their death. As of 2019, there were at least three anti-PD-1 and three anti-PD-L1 antibodies approved by the FDA for use in the clinic, along with additional antibodies undergoing clinical trials[55]. Targeting either receptor can allow for

enhanced targeting in combination with the immunotherapeutic properties, serving as a promising candidate for combinatorial nanocarriers with chemotherapeutic drugs. Yang et al. investigated the synergistic effects of anti-PD-1 and paclitaxel in a physical mixture fashion[56]. They began by observing the immunogenic cell death (ICD) effect of paclitaxel versus paclitaxel encapsulated by a previously generated methoxy-poly (ethylene glycol)-*b*-poly (D, L-lactide) (mPEG-PDLLA) nanocarrier, termed nano-PTX[56,57]. It was found that nano-PTX increased infiltration and activation of immune cells, but also resulted in increased PD-L1 expression within the tumor microenvironment. It was hypothesized that this overexpression of PD-L1 on the tumor cells caused by the PTX could be the cause of ICD-based treatment limitations. These issues were overcome when nano-PTX was followed by anti-PD-1 treatment, resulting in tumor regression and prolonged survival. This study demonstrates the feasibility of this combination strategy, namely concurrent delivery of nano-PTX and anti-PD-1 in the form of co-injection or physical conjugation of anti-PD-1 to the nanocarrier surface to enhance antibody delivery to the tumor site.

Lan et al. also used anti-PD-1 as their immunotherapeutic by conjugating it to the surface of a lipid nanoparticle loaded with cisplatin[58]. This approach demonstrated significant tumor regression. Furthermore, microneedles were employed to deliver the nanoparticles to the tumor site, which added to the synergistic effect of immunochemotherapy through the recruitment of T cells. Even without the added immune response caused by the microneedles, following the typical intravenous injection route of treatment is hypothesized to have also been efficacious against tumor growth. This could occur as a result of the blockade of PD-1 ligand on T cells combined with the cytotoxicity of cisplatin, both of which would be delivered directly to the tumor site through the passive targeting of the nanoparticle taking advantage of the EPR effect and active targeting via the anti-PD-1 antibody. Other groups have attempted combinatorial PD-1/PD-L1 immune checkpoint blockade by utilizing anti-PD-L1 antibody to target the PD-L1 receptor on the tumor cells.

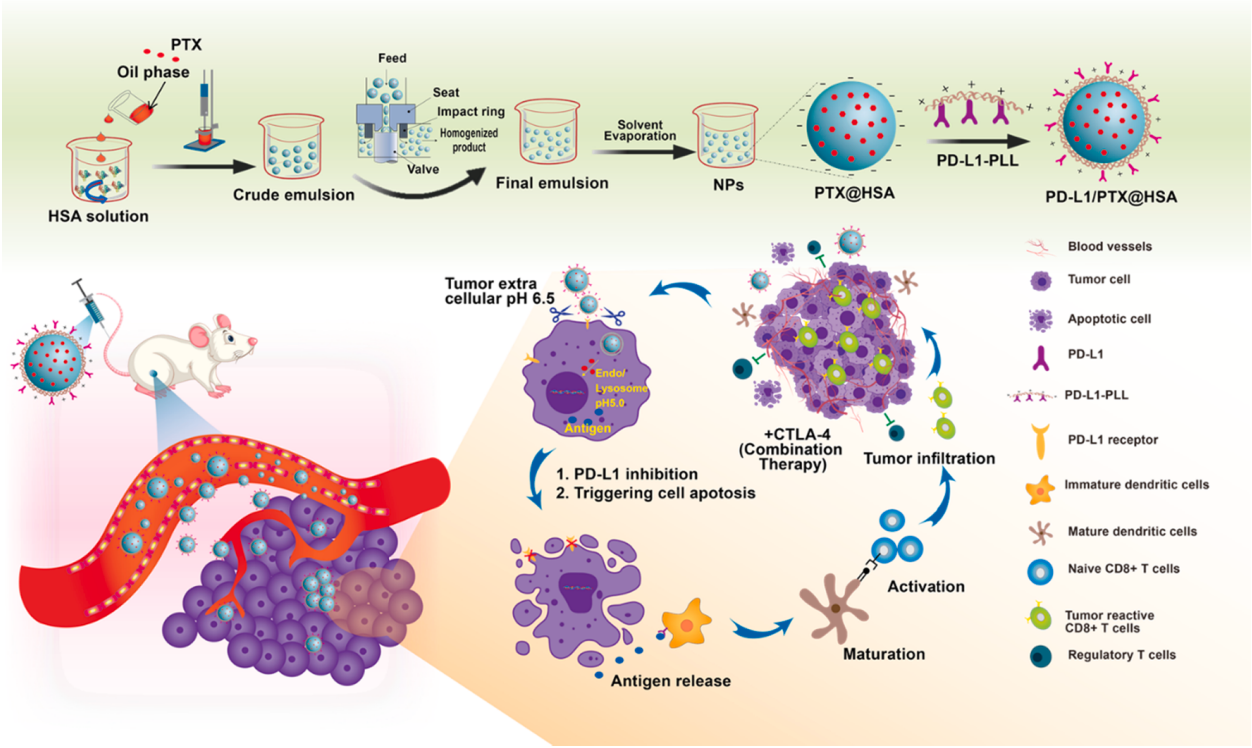

Figure 3. “Preparation and mode of action of PD-L1-targeted, HSA-loaded NPs with PTX for chemo-immunotherapy. HSA, human serum albumin; PTX, paclitaxel; PD-L1, programmed death-ligand 1; PLL, poly-L-lysine; NPs, nanoparticles.” Reprinted from International Journal of Pharmaceutics, 605, Le Minh Pham et al., Combination chemotherapeutic and immune-therapeutic anticancer approach via anti-PD-L1 antibody conjugated albumin nanoparticles, Pages No., Copyright (2021), with permission from Elsevier [59].

Pham et al. conjugated the anti-PD-L1 antibody to albumin nanoparticles loaded with paclitaxel(Figure 3)[59]. As with many drug-encapsulating delivery system formulations for chemotherapeutic delivery, the human serum albumin demonstrated increased drug release at lower pH values, similar to those of the tumor site instead of physiological pH. Anti-PD-L1-conjugated nanoparticles demonstrated enhanced cellular internalization and tumor accumulation when compared with their non-targeted counterparts. When this targeting and immune activation were combined with paclitaxel loaded into the nanoparticle, the treatment proved effective as demonstrated by tumor inhibition. They added a third synergistic component by treating with anti-CTLA-4 antibodies at the same time as the nanoparticle[59]. The triple combination exhibited the most enhanced effects among all the groups demonstrated by both infiltration of effector T cells and suppression of regulatory T cells as a result of both anti-PD-L1 and anti-CTLA-4 therapy, which was then compounded by the cytotoxic effects of paclitaxel. Important to note is that Pham

et al. investigated the off-target effects and biodistribution of the nanoparticle formulation, which caused minimal organ toxicity. This emphasizes the benefits of using nanocarriers for cancer treatment, taking advantage of the EPR effect and limiting the areas where these delivery systems can leave the bloodstream and have toxic effects.

Jiang et al. designed a hyaluronic acid-disulfide- D- α-tocopherol succinate micelle (HA-SS-TOS, HSST) that would target high CD44-expressing cancer cells[60]. While paclitaxel-loaded HSST micelles demonstrated inhibition of tumor growth and metastasis, they also ended up suppressing the immune system, caused by increased levels of TGF-beta, indicating a need for immune activation. They forwent the targeting ability of anti-PD-1 antibodies by encapsulating PD-1 antagonist peptides A12 within a PLGA microsphere[60-62]. Co-administration of both nanocarriers demonstrated effective inhibition of tumor metastasis in a lung metastasis tumor model, as well as increased survival in multi-drug resistant tumors[60]. While encapsulation of the A12 peptide proved effective, the A12@PLGA delivery system was only utilizing passive targeting. Despite the less immunotoxicity of free A12 peptide than anti-PD-1/PD-L1 antibodies, it undergoes rapid clearance[61]. This issue was solved by encapsulation, but the conjugation of the peptide to the nanocarrier surface would allow for active targeting and could potentially demonstrate similar clearance and synergistic benefits in combination with PTX@HSST.

Engineering a nanocarrier for dual chemo- and immunotherapy will heavily rely on the cancer type treated. Multiple cancers contain various tumor antigen expression levels and expression continues to rearrange the duration of treatment. Therefore, in order to maintain the efficacy of traditional chemotherapeutics, active and passive immunotherapies can synergize to target multiple pro-tumorigenic pathways.

4.0 Chemotherapy and gene delivery combination

Administration of conventional chemotherapeutics often leads to patients developing drug resistance. As a result, gene therapy can be used in combination in order to inhibit drug resistance or alternatively, work synergistically to inhibit multiple pro-tumorigenic pathways. Nanocarriers

can further improve this approach by targeting tumor sites and can offer simultaneous delivery of genes and cytotoxic drugs directly to cancer cells.

4.1 Targeting multidrug resistance

Cancer cells implementing continuous changes in their genome denotes a significant pathway used by cancer cells to perpetuate tumor survival. RNA interference (RNAi) can downregulate pro-tumorigenic genes such as P-glycoprotein, a drug efflux transporter, thus, disrupting tumor survival[63]. P-glycoprotein is an ATP-binding cassette (ABC) transmembrane transporter overexpressed on liver, ovary, breast, and brain cancer cells[64]. ABC transporters pump hydrophobic drugs like anthracyclines and mitotic inhibitors out in order to reduce intracellular drug concentrations[64]. Co-delivering genes, such as siRNA, with conventional chemotherapeutic drugs, can prevent cancer cells from altering their genetic code in order to develop drug resistance. Therefore, cells can be re-sensitized for effective prolonged chemotherapy treatment. Genes that are being chosen for cancer therapy include those encoding proteins involved in multidrug resistance, apoptosis (TRAIL, p53, TNF-alpha), and upregulation of cytotoxic cytokines (IL-12)[65].

The use of delivery systems facilitates effective systemic delivery of genetic material to cells as nanocarrier-mediated gene condensation prevents degradation via serum nucleases in blood. Without a gene carrier, an intramuscular injection of DNA only leads to a small fraction of successfully transfected target cells[66]. In the absence of cationic gene carriers, electrostatic repulsion would occur from the anionic genes with anionic plasma membranes. Therefore, nanocarriers are beneficial in providing cationic charges which can initiate intracellular delivery of genetic material.

Zhang et al. illustrated the benefit of a cationic gene vector for co-delivery of P-glycoprotein siRNA and doxorubicin using a triblock copolymer micelle containing N-succinyl chitosan–poly-L-lysine–palmitic acid (NSC–PLL–PA)[67]. These materials showed a pH-dependent release profile and demonstrated synergistic tumor inhibition *in vivo*[67]. Micellar delivery of doxorubicin alone using dendron-lipid micelles increased intracellular drug concentrations compared to free drug, improving the cytotoxic effect[68]. This result is observed because nanocarriers can deliver drugs via endocytosis, avoiding drug efflux pumps, therefore, delaying drug resistance[64].

However, cancer cells have the opportunity to develop resistance through an alternative pathway termed the anti-apoptotic defense mechanism[69,70]. The influx of chemotherapy drugs and genes can manipulate different cancer cell signaling pathways in order to prevent the development of multidrug resistance. The therapeutic effect of doxorubicin can therefore be further optimized through simultaneous downregulation of drug efflux transporters[67]. The NSC–PLL–PA based polymeric micelles for drug and gene co-delivery contained a favorable biodistribution profile, as the 170 nm particle size was appropriate to take advantage of the EPR effect[67]. The ability to target co-loaded micelles to the tumor site decreased off-target adverse effects, shown by a reduced loss of mice body weight compared to free drug alone[67].

The next generation of nanocarriers for drug-gene co-delivery includes the conjugation of targeting ligands to the surface[71-73]. Selecting a targeting ligand is dependent on the expression of the corresponding receptor of the target cell. In comparison to normal cells, cancer cells overexpress specific receptors; however, these receptors are not expressed homogeneously across the cell, and the binding affinity of targeting ligands to receptors is influenced by multiple factors[74]. Therefore, nanoparticle design must be carefully considered in order to optimize receptor binding. Jeong et al. engineered a PD-L1 binding peptide which when conjugated to G7 poly(amidoamine) dendrimers, showed 5-fold higher binding affinity to the binding counterpart, PD-L1 compared to free peptide[26]. The dendritic surface stabilized the peptide into a beta-hairpin structure via intermolecular forces and the excluded volume effect[26]. In addition to this, the hyperbranched structure of dendrimers can provide multivalency as multiple targeting ligands can simultaneously bind to multiple receptors at once, cooperatively enhancing binding[75]. Immunotherapeutics such as anti-PD-L1 antibodies have the benefit of acting as both targeting agents and immune checkpoint inhibitors. Although dendrimers can offer enhanced binding of targeting ligands, their capability of drug loading is limited. Dendrimers do not have an internal core that can retain encapsulation of drugs; therefore, drugs must be conjugated to the surface, complicating the nanoparticle design[76]. To combat this issue, a novel dendron-lipid micelle constructed from generation 3 poly(amidoamine) dendron and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was developed to encapsulate the drug doxorubicin while maintaining gene delivery properties[68].

An alternative drug carrier consisting of a cholesterol-modified peptide with micelle-like assembly could encapsulate cabazitaxel and condense inhibitory κB kinase (IKK) siRNA[73]. The

nanocomplex included a hyaluronic acid (HA) coating to target CD44 overexpressed on triple-negative breast cancer cells[73]. The hyaluronic acid increased cellular uptake 3-fold compared to non-targeted nanoplexes[73]. Interestingly, *in vivo* studies indicated that targeted co-encapsulation of cabazitaxel and IKK siRNA significantly inhibited tumor growth compared to the delivery of targeted nanocomplex containing siRNA and free cabazitaxel[73]. Passive and active targeting increases the delivery of drugs specifically to tumor sites, decreasing drug accumulation in non-target cells[77]. Co-encapsulation of drug and genes allows for efficient simultaneous delivery to cancer cells, disrupting multiple tumorigenic pathways and yielding synergistic effects in tumor inhibition[71].

4.2 Targeting cancer immune evasion

Numerous studies have aimed to deliver genetic material in order to avoid multidrug resistance for a delivered chemotherapeutic[67,78,79]. However, genes related to the activation of immune cells within the TME can also be used in order to incorporate cancer immunotherapy. As previously mentioned, antibody-based immunotherapies are not effective for patients with "cold" tumors[80]. However, immune-related gene delivery can re-modulate the TME, opening the opportunity for effective cancer treatment. Li et al. achieved this by using human serum albumin containing a CXCR4 antagonist, low dose of paclitaxel and PD-L1 siRNA to improve immunotherapy[81]. The interference of the chemokine network, specifically the CXCL12/CXCR4 axis, can reduce tumor fibrosis, allowing for more effective migration of $CD8^+$ T cells within the TME[82]. To further optimize immune activation, a low dose of paclitaxel was incorporated to promote calreticulin (CRT) exposure on cancer cells, activating local dendritic cells, thus, re-sensitizing the immunosuppressive TME[81]. This study shows the benefit nanocarrier-mediated drug and gene co-delivery has to enhance the therapeutic efficacy of cancer chemoimmunotherapy.

5.0 Gene therapy and immunotherapy combination

Besides immune cells influencing the immunosuppressive nature of the TME, hypoxia also plays a role[83]. The acidic TME is maintained by the continuous production of lactate from cancer cells under hypoxic conditions, making tumor-infiltrating cytotoxic T lymphocytes (CTL) anergic and apoptotic[84]. On the other hand, Tregs have a metabolic advantage in the lactate-rich

microenvironment, sustaining their survival[85]. For this reason, Zhang et al. constructed a nanoplatform with the ability to reverse CTL anergy using lactate dehydrogenase siRNA which also enhanced anti-PD-1 treatment[84]. A cationic lipid-polymer model was used consisting of *N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-(2-cholesterloxycarbonyl amino ethyl) ammonium bromide (DOTAP) to bind to negatively charged siRNA and poly(ethylene glycol)-block-poly(lactide-co-glycolide) (PEG-PLGA) to increase circulation time while reducing clearance by the reticuloendothelial system (RES)[84]. Interestingly, this group compared the *in vivo* efficacy of their bilayer and monolayer vesicles (micelles) for siRNA delivery and found the bilayer vesicles exhibited longer blood circulation, slower release of siRNA, and higher tumor accumulation of siRNA[84]. This result was attributed to the ability to protect genes via encapsulation compared to complexation on the external surface. When lactate dehydrogenase knockdown was combined with anti-PD-1 treatment, a synergistic effect was obtained inhibiting 68.2% of tumor growth compared to no significant changes from either treatment alone[84]. Decreasing Treg populations by 60% using lactate siRNA can be owed to the enhanced tumor inhibition observed as the immunosuppressive TME was remodeled[84]. Although synergistic results were obtained by this co-delivery approach, there is an opportunity in potentially enhancing the therapeutic outcome by creating a single drug carrier for the simultaneous delivery of lactate siRNA and anti-PD-1. To optimize the combination of gene therapy with immunotherapy, other gene targets and their synergistic roles in immunotherapies will need to be studied. Further commercialization of this combination approach can lead to the personalization of cancer treatment as the selection of a gene target to manipulate may be patient specific.

Table 2. Advantages and disadvantages of various approaches for chemoimmunotherapy[86].

Approach	Advantages	Disadvantages
Free drug + immunotherapy nanocarrier	1. Easy synthesis and scale-up 2. Adjustable dosage	1. Off-target effects 2. Unwanted biodistribution 3. Poor tumor targeting 4. Potential systemic toxicity
Drug nanoparticle + immunotherapy nanocarrier	1. Adjustable dosage	1. Differing biodistribution and tumor accumulation 2. Differing clearance and pharmacokinetics
Co-encapsulation	1. Simultaneous spatial delivery 2. Uniform distribution 3. Correct tumor accumulation ratio 4. Controlled release	1. Complex preparation 2. Best for single target
External immunotherapeutic conjugation + encapsulated drug	1. Enhanced targeting and cellular uptake 2. Easy preparation	1. Immune response outside tumor

	3. Simultaneous spatial delivery 4. Uniform distribution 5. Correct tumor accumulation ratio 6. Controlled release	

6.0 Clinical trials

Treatment of cancers have long suffered from the issues of multidrug resistance, systemic toxicity, and relapse. The use of chemoimmunotherapeutics and combination nanomedicines works to solve these problems, with some promising systems translating into the clinic. Many clinical trials investigating chemoimmunotherapeutics are in phase one and two, with numerous of these trials focusing primarily on metastatic triple negative breast cancer[87]. Some problems that have arisen in trials are the inability for patients receiving higher doses of the chemoimmunotherapeutic being unable to complete the full treatment regimen. For example, patients with stage IIIB cervical cancer that received the "level 3" dose did not complete the full protocol, with leukopenia serving as the limiting factor[88]. Typical treatment strategies simply rely on co-injection of chemo- and immunotherapeutics, not taking advantage of the passive targeting afforded by conjugation or encapsulation with nanocarriers. Chemoimmunotherapy clinical trials that are currently recruiting or underway tend to use the same nanoparticle formulation, albumin-bound paclitaxel. Nab-paclitaxel, a paclitaxel drug covalently bound to an albumin molecule, which is naturally occurring within the body, is an FDA-approved therapeutic. The immunotherapeutics chosen for the clinical studies are all monoclonal antibodies approved by FDA (**Table 3**). Each of the clinical trials is utilizing the "free immunotherapy + drug-nanocarrier" approach with the hopes of increased efficacy from the combinations. Chemoimmunotherapy nanomedicine that utilizes the other combination approaches are in pre-clinical trials. The novel delivery systems in **Table 4** are examples of the future direction of treatment options for cancer patients.

Table 3. Current clinical trials combining chemotherapy and immunotherapy nanomedicine.

Nanocarrier Formulation	Immunotherapeutic	Chemotherapeutic	Disease State	Reference / Identifier
Albumin-bound paclitaxel	Durvalumab	Paclitaxel	Stage III non-small cell lung cancer	NCT05157542
Albumin-bound paclitaxel	Camrelizumab	Paclitaxel	Gastric cancer	NCT05101616
Albumin-bound paclitaxel	Ipilimumab Nivolumab Pembrolizumab	Carboplatin Paclitaxel Pemetrexed	Advanced lung cancer	NCT04929041

Albumin-bound paclitaxel PEGylated liposomal doxorubicin hydrochloride	Durvalumab	Capecitabine Carboplatin Gemcitabine hydrochloride Paclitaxel Doxorubicin hydrochloride	Advanced malignant solid neoplasm	NCT03907475
Albumin-bound paclitaxel	Pembrolizumab	Paclitaxel Pemetrexed Carboplatin	Non-small cell lung cancer with brain metastases	NCT04964960
Albumin-bound paclitaxel	Nivolumab Ramucirumab	Cabozantinib S-malate Docetaxel Gemcitabine hydrochloride Paclitaxel	Advanced non-squamous non-small cell lung cancer	NCT04310007
Albumin-bound paclitaxel	Durvalumab Oleclumab	Gemcitabine Paclitaxel	Stage I pancreatic cancer	NCT04940286
Albumin-bound paclitaxel	Durvalumab Tremelimumab Personalized synthetic long peptide vaccine	Carboplatin Gemcitabine hydrochloride Paclitaxel Poly ICLC	Stage IV, invasive, and metastatic breast cancer	NCT03606967
Albumin-bound paclitaxel	Atezolumab Bevacizumab	Cobimetinib Paclitaxel Vemurafenib	Advanced thyroid gland cancer	NCT03181100

Table 4. Examples of chemoimmunotherapy nanomedicine in pre-clinical trials[86,89].

Nanocarrier Formulation	Combination Approach	Immunotherapeutic	Chemotherapeutic	Cell line / tumor model	Reference / Identifier
Liposome	Co-encapsulation	PD-L1 inhibitor	Doxorubicin	B16F10 tumor-bearing C57BL/6 mice	[90]
Liposome	Co-encapsulation	Anti-PD-L1	Docetaxel	B16F10 xenographic tumor model	[91]
Polymeric micelle	Co-encapsulation	Anti-PD-L1	All-trans retinoic acid	C3H tumor-bearing mice	[92]
Polymeric micelle	Co-encapsulation	Anti-DR5	Dacarbazine	A375 and NIH cells A375 BALB/c nude mouse tumor model	[93] [94]
Polymeric micelle	Co-encapsulation	HY19991	Paclitaxel	MCF-7 tumor-bearing mice	[95]
Polymeric micelle	Co-encapsulation	NLG919	Curcumin	B16F10 tumor-bearing C57BL/6 mice	[96]
Polymeric micelle	Co-encapsulation	Anti-PD-L1	Paclitaxel	B16F10 tumor-bearing C57BL/6 mice	[97]

Polymeric micelle	Drug encapsulation + siRNA complexation	Snail siRNA (siSna) Twist siRNA (siTwi)	Paclitaxel	4T1 breast tumor model	[98]

7.0 Expert opinion

The insolubility and hydrophobic nature of chemotherapeutics require cytotoxic solvents for dissolution and subsequent injection, thus increasing the cytotoxicity of already cytotoxic drugs. The inherent lack of cell specificity of small molecule drugs increases occurrences for side effects which support patients to prematurely discontinue treatment[65]. This problem is effectively negated by encapsulation within nanocarriers for release only once inside the tumor microenvironment. The use of nanocarriers in cancer treatment will allow for decreased systemic toxicity, by specifically directing immunotherapeutics, drugs, and/or genes to the tumor site. Although controversial, this is possible simply by passive targeting via the EPR effect. Furthermore, the addition of targeting ligands on the nanocarrier surface can further improve tumor specificity of the delivery systems and reduce toxicity attributed to off-target effects. The precise control based on both passive and active targeting would ultimately enable us to engineer tailored nanocarriers that individually address the need from each patient. As cancer therapy evolves, personalized medicine will become routine, tailoring treatments to a patient's specific cancer cell type.

The TME is consistently reconstructing itself as it adapts to new anti-cancer therapies. The continuous development of immunotherapy opens a new perspective on how we can best achieve remission for cancer patients. The original viewpoint on cancer treatment has stood from the idea of continuous discovery and development of new anti-cancer agents that will lead us to the discovery of a superior small molecule drug with effective anti-cancer capabilities. However, this idea is not realistic as we continually learn that tumors are complex and cancer cells are dynamic, always mutating against any anti-cancer agent in order to maintain survival. Revisiting the hallmarks of cancer reminds us that one anti-cancer agent is unlikely to maintain its efficacy across all patient populations. Combination approaches, more specifically chemoimmunotherapies have demonstrated that synergistic outcomes in tumor inhibition can be achieved. Their success is partially attributed to conventional chemotherapeutics causing ICD, releasing damage-associated

molecular pattern (DMAP) molecule that triggers recruitment of dendritic cells and sequential priming of CTLs, promoting a direct cytotoxic response of T cells to cancer cells[99,100]. The success behind combination approaches is significant and critical in meeting the goal of tumor elimination across numerous tumor types. Combination therapy gives access to personalized medicine, where each patient's cancer treatment can be tailored to their tumor microenvironment conditions. However, simply mixing the different therapeutic agents would not be effective.

Continuous development of nanocarrier-mediated delivery of chemoimmunotherapies will optimize this combination approach by offering preferred biodistribution, toxicity, and efficacy profiles. Nanocarrier design must be tailored to optimize the delivery of the intended combination approach. Creating a combination approach within a single nanocarrier means that a multifunctional nanocarrier must be developed while considering numerous factors regarding biological interferences. Maintaining a size large enough to take advantage of the EPR effect while also small enough so the final nanocomplex is small enough to penetrate the dense extracellular matrix of solid tumors[13]. Understanding the dynamic TME creates considerations on which target genes, chemotherapeutics and targeting ligands to functionalize a nanocarrier. Wu et al., understood this by co-delivering doxorubicin with an immune cocktail containing PD-L1 siRNA and a plasmid expressing the ECM destroyer, hyaluronidase[101] (2021). Doxorubicin and PD-L1 blockade have been shown to synergistically enhance immune activation through ICD and checkpoint blockade; therefore, hyaluronidase expression allows efficient trafficking of T cells into the TME[101]. Researchers have also made efforts to improve immunotherapy through the modulation of the cytokine network. Inducing cancer cells to express anti-tumorigenic molecules such as interleukin-2 (IL-2) can drive T cell expansion and infiltration[102]. Ideally, a trimodal approach (drug, gene, and immune checkpoint inhibitor) would be the optimal combination approach because chemotherapies and gene therapies can work synergistically with immunotherapies to restore immunosurveillance. Using nanocarriers can improve the therapeutic outcome for combination approaches because they tune the delivery of all therapeutic agents into one singular biodistribution profile[102].

A crucial component in engineering a multifunctional NP is ensuring all functionalities can be done efficiently. The gene complexation capabilities of liposomes decrease from 92% to 20% when co-encapsulating imatinib[103]. While co-encapsulating Nile red and DNA plasmid using generation 3 dendron-lipid micelles showed no effect in gene complexation efficiency[68]. Multifunctional nanocarriers must also maintain a balance between acquiring properties for the proton sponge effect while keeping biocompatibility. Excess cationic charges can not only induce cytotoxicity but instability as well due to serum protein adsorbing to the surface, destabilizing the gene-nanocarrier polyplex. To combat this issue, PEG and anionic coatings can be used to shield the remaining excess cationic charges[72]. An efficient multifunctional delivery system will have gene and drug loading capacity, lysosome escape, stability, biocompatibility, and tumor targeting. In the future, studies must determine the optimal sequential release of drugs and genes which result in effective synergistic outcomes. This information would support the development of a nanoplatform with a controlled co-delivery approach[72].

Combination nanomedicine is already present in pre-clinical experimentation, with limited formulations currently in clinical trials. The idea of nanocarrier-mediated delivery of chemotherapeutics has already been proven by the FDA-approved drug, Genexol-PM®, composed of paclitaxel encapsulated by an mPEG-PDLLA polymeric micelle. Specific immunotherapy antibodies are also approved, such as Trastuzumab, and the combination of these treatments is currently in review in clinical trials with the co-encapsulation approach in pre-clinical trials. Based on this, we can anticipate that nanocarrier-mediated co-delivery of chemoimmunotherapies is on the horizon to be implemented in clinical practice. One barrier to overcome is creating a combination therapy that can be upscaled in an affordable and easy way. Also, the storage stability of combination approaches must be carefully considered as these platforms can involve unstable components such as easily degradable siRNA. Consistent drug encapsulation during storage must also be determined as the premature release of drugs will decrease efficacy. To overcome these barriers, covalently crosslinking micelles can prevent micelle dissociation and maintain drug encapsulation[104]. Achieving formulations that can be stored as powders can potentially alleviate concerns for shelf stability. However, obtaining freeze-dried powders can be difficult as studies show lyophilizing micelles increases their size, therefore, altering their biodistribution[105].

Studies determining cryoprotectants will be required to ensure minimal size and polydispersity changes during the lyophilization process.

Today standard procedure for cancer treatment still relies heavily on chemotherapy, radiation, and surgery. As we move towards personalized medicine where carefully selected combination approaches are prepared, treatment of cancer patients with monotherapies will be seen less frequently. Dually attacking cancer cells using an inhibitor cocktail (anti PD-L1, anti PD-1, CTLA-4) with chemo and genes can dually attack multiple pro-tumor pathways, making it difficult for cancer cells to adapt and maintain survival by adapting multiple pathways at the same time.

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