Nanoparticle-based combination drug delivery systems for effective cancer treatment

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Chapter 7

General discussion

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The widespread application of nanomedicine has broken many limitations of traditional cancer therapies [1]. The focus of current research is designing different kinds of nanomaterials to meet various medical purposes. This thesis aims to explore combinational drug delivery systems based on nanoparticles in cancer treatment, particularly investigating the feasibility of integrating imaging and treatment modalities, such as immunotherapy based on regulating TAM and inhibiting CAF activity. Due to significant knowledge gaps in combinational therapies utilizing rare-earth NPs and chemotherapeutic drugs, in **Chapter 2**, the physicochemical properties, cell toxicity, and fluorescence characteristics of multifunctional drug-loaded CaF_2:Y, Nd+DOX@PLGA/PEG/EGF NPs for synergistic therapy and imaging were fully tested and analyzed. Building upon this foundation, our research extends into tumor immunotherapy. The TME presents a major challenge in current immunotherapies, particularly due to the tumor-promoting effects of TAMs and CAFs. To inhibit the tumor-promoting properties of M2-TAMs, we implemented a series of validation experiments to explore whether lipids can promote the repolarisation of M2-TAM into M1-TAM, and whether they can inhibit tumor cell proliferation and invasion, and whether lipids encapsulated in NPs can inhibit the tumor-promoting properties of TME *in vitro* and *in vivo* (**Chapter 4 and 5**). To explore the effect of drug-loaded nanoparticles on CAF activity during cancer treatment, a 3D FTM simulating SKCM and cSCC was developed to examine the tumor penetration potential, tumor killing potential and modulation of CAFs by NPs (**Chapter 6**). This chapter provides a general discussion of the overall research effort, analyzing the potential causes and implications for future research.

Objective 1: To develop novel multifunctional NPs for simultaneous monitoring and therapy by enhancing cellular uptake in tumor cells.

Rare earth elements possess exceptional optical properties. In **Chapter 2**, we synthesized CaF_2: Y, Nd NPs by a hydrothermal method and evaluated their physical and chemical properties to determine their potential as fluorescent probes. The results showed that CaF_2:Y, Nd NPs have typical properties of fluorescent probes, such as homogeneous particle size, stable crystal structure, stable optical properties [2], extremely narrow emission line [3], and high sensitivity [4]. Subsequently, we encapsulated CaF_2: Y, Nd NPs and DOX within PLGA-PEG-EGF using double emulsion to form CaF_2: Y, Nd + DOX@PLGA/PEG/EGF NPs. However, uncertainties persisted regarding whether this synthesis would affect the optical properties of CaF_2: Y, Nd NPs, induce cell toxicity, enhance cellular

absorption, or compromise the efficacy of DOX. Therefore, we conducted analyses to determine whether CaF_2: Y, Nd + DOX@PLGA/PEG/EGF NPs retained the therapeutic and fluorescent imaging properties of the encapsulated compounds. It was found that the original optical properties of CaF_2:Y, Nd NPs were minimally affected by the addition of DOX and PLGA-PEG-EGF [1]. CaF_2: Y, Nd + DOX@ PLGA/PEG/EGF NPs exhibited increased uptake by cancer cells, likely attributable to the presence of EGF, while DOX contributed to cancer cell death. However, compared to CaF_2: Y, Nd + DOX@PLGA/PEG NPs, EGF-modified NPs did not exhibit a significantly increased uptake by cancer cells, which prompts consideration of other factors affecting NP uptake. For instance, EGFR on cancer cells may be saturated with endogenous EGF, or NPs may bind non-specifically to the cell membrane, or NPs may not effectively target receptors on the cell surface [5]. The formation of protein corona in organisms has been reported to encapsulate pre-coupled targeting ligands, thereby blocking ligand-receptor recognition, and the smaller the ligand the greater the chance of being affected [6]. In addition, the specific properties of NPs, the concentration of NPs used, and the exposure time can all affect the uptake of NPs [7]. Many issues still exist regarding the optimization of NP uptake and enhancement of therapeutic efficacy by coupling EGF. The addition of ligands, receptors, antibodies or other modulating agents to enhance or synergize the therapeutic effect is currently being explored [8]. In this context, our study aimed to enhance NP uptake by coupling EGF and monitoring distribution and accumulation using the optical properties of rare earth elements in NPs. This approach offers a novel direction for optimizing drug delivery, controlling drug dosage, and improving cancer therapeutic outcomes.

Objective 2: Exploring the role of lipids in regulating tumorigenicity and TAM repolarisation in 4T1 breast cancer cells.

In **Chapter 3**, we extensively discussed the current state of research on tumor immunotherapies targeting TAM-promoted tumor phenotypes within the TME. We analyzed the significant potential of various immune therapies aimed at TAMs in both preclinical and clinical studies [9]. Additionally, a clinical trial at Radboud University Medical Centre (NCT03397238) proposes a cancer treatment strategy to control TAM functional polarisation by reprogramming before TME recruitment. However, there remains a notable research gap in effectively reprogramming tumor-promoting TAMs within the TME to develop immunotherapy against them. Lipids have been reported to induce repolarization of M2-TAMs and play a crucial role in tumor cell proliferation and migration by modulating cellular uptake and fatty acid oxidation [10,11]. These findings underscore the potential utility of

lipid-based interventions in reshaping the TME and enhancing the efficacy of immunotherapeutic strategies targeting TAMs.

In **Chapter 4**, we investigated the effects of four lipids—PA, SM, Cer, and DHA—in breast cancer treatment and TAM polarization regulation, aiming to identify lipids capable of reversing pro-tumor M2-TAMs to anti-tumor M1-TAMs while inhibiting tumor lipid metabolism. To mitigate potential macrophage polarization responses or lipid toxicity-related side effects, we treated tumor cells with low lipid concentrations. Although these low lipid concentrations did not affect cancer cell activity, they effectively inhibited the proliferation and migration of breast cancer cells, indicating the ability of the selected lipids to inhibit cancer cell migration, consistent with previous research. PA has been reported to inhibit cancer cell proliferation by sensitizing MCF-7 cancer cells or delaying the cell cycle [12,13], Cer could inhibit MCF-7 cancer cell proliferation by blocking the cell cycle [14], and DHA inhibited cell proliferation of 4T1 and MCF-7 cells by promoting cell cycle arrest [15,16]. In addition, low concentrations of PA, DHA and Cer reduced the phenotype of M2-type macrophages, and the phenotype of M2-TAM decreased significantly after PA and Cer treatment. However, SM had minimal effect on the M2-TAM phenotype. It's important to note that the effect of different lipids on M2-TAM repolarization is complex and dependent on factors such as lipid type, concentration, and metabolic pathways involved. For instance, Omega-3 fatty acids, such as DHA and eicosapentaenoic acid, have been shown to inhibit the STAT3 and NF-κB pathways involved in M2 polarisation, while activating the JNK and p38 pathways involved in M1 polarisation [17]. In contrast, omega-6 fatty acids, such as arachidonic acid , have been shown to promote the M2 phenotype [18]. The mechanisms underlying lipid-induced macrophage repolarization are not fully understood, necessitating further research into lipid receptors, signaling and metabolic pathways, and epigenetic modifications. Thus, by elucidating the mechanisms underlying lipid-induced M2-TAM repolarization, we may develop new strategies to promote this process in modern cancer therapies.

Objective 3: To explore the effect of lipid-based NPs on TAM repolarisation and the potential for treatment of breast cancer in mice.

Previous research has demonstrated that PA not only directly affects the proliferation and invasion of breast cancer cells, but also reduces the pro-tumorigenic properties of M2-TAMs by promoting their repolarization, which provides the rationale for exploring the effect of PA *in vivo*. However, PA presents issues such as low water solubility, poor cell permeability, weak tumor-killing

ability, and poor tumor selectivity. To overcome these challenges, in **Chapter 5**, we used non-toxic PLGA as a nanocarrier to encapsulate PA and DOX , aiming to comprehensively investigate the effects of these NPs on tumor growth and TME *in vivo* and *in vitro*. By analyzing the release profiles of DOX and PA from the NPs, we found that DOX was released rapidly from the NPs, while the release rate of PA was slower. The release rate of a drug directly affects its therapeutic efficacy. In certain cases, a rapid release of the drug from the NP may be advantageous, such as when high concentrations of drugs are required at the target side for therapeutic efficacy, then a rapid release from the NP may be advantageous. However, rapid drug release may not be ideal in other circumstances, particularly if the drug is toxic or has a narrow therapeutic index, which can lead to toxicity and adverse effects [19]. In addition, if the drug is intended to have sustained release properties to provide extended therapeutic effects, then rapid release of the drug from the NP is not appropriate [20]. In this study, the rapid release of DOX effectively killed tumor cells, followed by the slower release of PA, which acted on TAMs surrounding the tumor cells, aligning with our expectations. This is in line with our expectations. In summary, NPs should be designed with careful consideration of their intended application and physicochemical properties, including drug release profiles, to optimized to maximize the therapeutic effect. PA-loaded NPs demonstrated the ability to promote the repolarization of M2-TAMs to M1-TAMs *in vitro*, indicating a potential immune-stimulating effect.

In vivo studies demonstrated that PA-loaded NPs induced lymphocyte infiltration and reduced the immunosuppressive properties of TME, along with the expression of M2-TAM markers. However, no significant changes were observed in the expression of M1-TAM markers. When this occurs, we should consider the complex environment *in vitro* and *in vivo*. Firstly, *in vitro* studies were performed on isolated cells in a controlled environment, whereas *in vivo* studies involve complex interactions between different cell types and tissues. This situation suggests that the effects of PA on macrophage polarization may differ in complex and dynamic environments such as the TME, which may be affected by factors such as tumor development stage or the overall immune status of the host [21]. Secondly, the effect of PA on immune cells may also not be limited to macrophages, and other immune cells or factors may also contribute to the observed effects on TME [22]. Therefore, further studies are needed to fully understand the mechanisms underlying the effects of PA-loaded NPs on TAM polarization and immune cell activity in the TME, which may provide a new strategy for targeting anti-cancer therapies to TAM and tumors.

Objective 4: To explore the therapeutic potential of NPs containing chemotherapeutic agents for skin cancer under the condition of existing cancer-associated fibroblasts (CAFs) *in vitro*.

CAFs, as the main component of the TME, play a crucial role in cancer growth by promoting tumor cell proliferation, and invasion, and inducing drug resistance. To understand the complexity of different TME cell subpopulations, in **Chapter 6**, we explored the impact of NPs on tumor growth in the presence of CAFs in a skin cancer model. To discern the potential effects of NPs on various cell types in the skin cancer microenvironment, we evaluated their impact on cell viability using skin cancer cell lines (melanoma and cSCC cell lines) and CAFs. Our analysis revealed distinct sensitivities of CAFs and skin cancer cell lines to the same type of nano drug, with CAFs requiring a higher dose of NPs, indicating their reduced sensitivity to NP cytotoxic effects. This difference can be attributed to the disparate cellular characteristics and functions between CAFs and skin cancer cells. While CAFs, as non-cancerous cells in the TME, support tumor growth and metastasis [23], cSCC and melanoma cells exhibit distinct metabolic and signaling characteristics, influencing their response to NP uptake and toxicity [24]. Moreover, NP uptake by CAFs differs from that by skin cancer cell lines due to variations in cell surface receptors, membrane permeability, cellular metabolism, and intracellular transport mechanisms. For instance, cells with higher metabolic activity tend to uptake more NPs [25]. Davis *et al*. have also discussed in depth how to design NPs to selectively target cancer cells based on the expression of specific receptors [26]. Behzadi *et al*. showed that the amount of NPs taken up by cells is determined by specific lysosomal and endosomal pathways in the cell, and cells with more lysosomes may be more effective in degrading NPs, while cells with more nuclei may be better at recycling them [7]. These research results help to develop personalized medicine, that is, designing more effective treatment plans targeting specific cell types based on a patient's specific cancer type and their sensitivity to drugs. However, the TME is a complex whole, and the different responses of different cell lines to the same type of nano drug may make it challenging to determine the optimal clinical dosage of drugs. Targeting CAFs with higher NP doses may result in severe side effects or toxicity in normal cells or tissues, complicating the interpretation of clinical trial outcomes.

To further analyze the effect of nano chemotherapeutic agents on tumor proliferation and apoptosis in the presence of CAFs, a 3D FTM simulating SKCM and cSCC was developed. However, dual targeting of NPs based on tumor cells and CAFs has been rarely studied in skin cancer. To explore the therapeutic potential of NPs in skin cancer models, we evaluated their tumor penetration,

tumor-killing effect, and regulation of CAFs through both topical and systemic administration. Notably, the next study was more challenging as CAFs and skin cancer cell lines differ in their sensitivity to chemotherapeutic agents, and here the concentrations of NPs were set according to the sensitivity of skin cancer cell lines to NPs. Although low concentrations of NPs can inhibit the growth of tumor cells, it may not effectively inhibit the pro-tumor properties of CAFs thereby failing to achieve the goal of inhibiting skin tumor growth. Therefore, we have conducted an in-depth study of tumor cells and CAFs in skin models. The results showed that DOX@PLGA NPs not only inhibited the proliferation and invasion of skin cancer cells in SKCM and cSCC, but also inhibited the activation of CAFs. These results underscore the significant potential of NPs for dual targeting of cancer cells and the TME, offering a promising avenue for the development of novel and effective skin cancer therapies. Importantly, no significant difference in therapeutic efficacy was observed between the two dosing modalities, suggesting their interchangeability in certain scenarios. This flexibility provides clinicians with additional options for treatment [27].

FTM serves as a controlled *in vitro* system that offers a high-throughput screening tool for the development of nanomedicines [28], allowing researchers to evaluate various formulations, doses, and delivery methods for their safety and efficacy against skin cancer cells in a controlled environment. Additionally, it aids in gaining a deeper understanding of the behavior and response of tumor cells within a three-dimensional setting. In this study, FTM effectively simulated the coexistence of CAFs and skin cancer, providing an ideal environment for an in-depth investigation into the interaction between CAFs and tumor cells. However, FTM does have limitations in accurately replicating the complex microenvironment of skin cancer *in vivo*, including interactions with the immune system, stromal cells, and the extracellular matrix. Therefore, further studies utilizing real skin or novel models that mimic real skin are necessary to validate the findings obtained from the FTM system.

Future perspectives

A growing body of research has highlighted the importance of modulating the TME to achieve successful tumor eradication, emphasizing its pivotal role in cancer therapy. Recently, much research has focused on disrupting and reprogramming TME, such as modulating TAM and CAF. For example, Zhang *et al*. developed bionic anti-cancer NPs using Toll-like receptor properties to reprogram M 2 macrophages with anti-tumor properties [29]. Despite the great progress made,

there are still many unanswered questions, such as how to effectively modulate TME, the specific molecular mechanisms driving TAM reprogramming, and their link to the various stages of cancer progression, which determine the feasibility and effectiveness of the drugs or approaches used and are key to solving the problem.

In this thesis, we initially investigated the impact of lipids on reprogramming M2 macrophages, laying the groundwork for combining immunotherapy and chemotherapy in breast cancer treatment. Both the type and concentration of lipids were found to influence breast cancer cells and M2 macrophages. The experimental data in **Chapter 4** showed that low concentrations (≤ 30 μM) of lipids could induce anti-tumor properties in M2 macrophages without affecting their activity. Although M2 macrophages exhibit antitumor properties in the presence of lipids, we are optimistic about the medical future of lipids as this study is not a thorough study of the molecular mechanisms of repolarisation of M2 macrophages by lipids and is at a preliminary stage of research, but its experimental results are satisfactory.

Recently, nanotechnology-based therapeutic options have garnered increasing attention, particularly in cancer treatment. Numerous nanomedicines are either undergoing clinical trials or awaiting approval, with some drug carriers, such as PLGA, already FDA-approved [30]. However, the development of nanomedicines presents more challenges compared to traditional drug development alone. Issues such as controlling drug loading, optimizing the drug-to-carrier ratio, and scaling up production while maintaining consistency pose significant hurdles. Despite these challenges, the potential of nanomedicines is undeniable. For instance, doxorubicin, a widely used broad-spectrum antitumor drug, is associated with severe side effects including cytotoxicity and cardiotoxicity [31]. Although clinically effective, the severe side effects forced patients to stop using it. The advent of nanomedicine has brought new hope to cancer patients. Scientists have used nanotechnology to design NPs containing DOX, and the results of many experiments in mice show that the side effects caused by DOX are greatly improved. Consequently, nanomedicine holds promising prospects for enhancing drug properties, improving bioavailability, and minimizing adverse effects in future clinical applications.

Although NPs have many advantages as drug delivery systems, the tracking of drug distribution, controlling drug dosage and improving the utilization of nanodrugs are still the focus of current research. With the advent of fluorescent probes, it is possible to track drug distribution and detect drug dosage, and RENPs are promising for drug tracking and fluorescent imaging due to their superior optical properties. The uptake of NPs by the organism is mainly divided into passive

targeting (EPR effect) and active targeting (ligand-receptor binding). However, to date, the existence of the EPR effect and whether it is effective are unanswered questions. Active targeting appears to be a more valuable therapeutic option than passive targeting. As research in nanomedicine advances, the study of targeted NPs is receiving increasing attention. Two important aspects underlying the development of targeted NPs are specificity and effectiveness. For example, targeted immunotherapy aims to achieve tumor suppression by modulating the body's immune system. However, there are many different types of immune cells, and the precise targeting of highly expressed receptors on cells that have an inhibitory effect on tumor growth is critical to the success of active targeting. Therefore, modifying the surface of target cells with highly specific and highly expressed receptors or ligands by surface modification of NPs is the current challenge and prospect of active targeting research.

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