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Gel-based drug delivery for safe and effective cancer immunotherapy

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CHAPTER 7

General discussion

Chih Kit Chung

1. Summary of the results

This chapter reflects on the research from this thesis and the conclusions that can be drawn in each study. Cancer immunotherapy research focuses on designing therapies that can stimulate the body's immune system to mount an effective immune response against the tumor. Under specific conditions, patients respond well to immunotherapy and the field of research is making impressive leaps forward. However, there are also concerns about the induction of side effects. In the last decade, researchers have spent tremendous effort to optimize the delivery and release rates of immunotherapeutic drugs with the help of many drug delivery systems. There is increasing belief that controlling drug release rates and location could mitigate side effects. This thesis focused on optimizing the delivery of immunotherapeutic drugs with the help of an injectable poloxamer 407 (P407) and an implantable hydrogel based on bacterial cellulose (BC). The main emphasis was to improve checkpoint blocking immunotherapy, but two chapters were also dedicated to photodynamic therapy (PDT) and chemotherapy.

The emphasis of the first chapters was preparing and characterizing an injectable thermosensitive P407 hydrogel formulation. In **Chapter 2**, P407 hydrogels ranging from a concentration of 14% to 40% were prepared. The aim of this first study was to prepare a P407 gel formulation that could slow down anti-CTLA-4 antibody release. It turned out that the 25% P407 formulation was the most favorable formulation for in vivo application. 25% P407 hydrogels were easy to inject and formed a gel rapidly at body temperature. In vitro release studies demonstrated that the gel released all antibodies within seven days. Cell culture studies revealed that P407 neither induced dendritic cell (DC) maturation nor exerted cytotoxic effects, indicating that the material was free from endotoxins and not immunogenic. Subsequently, anti-CTLA-4 loaded P407 hydrogels were injected in mice and serum analysis revealed that P407 hydrogels significantly reduced anti-CTLA-4 levels. Furthermore, P407 hydrogel based anti-CTLA-4 therapy resulted in efficient tumor growth inhibition in the CT26 and MC38 model. This effect was comparable with anti-CTLA-4 given in PBS or Incomplete Freund's Adjuvant (IFA). The serum antibody levels were lower than in the mice treated with PBS anti-CTLA-4, but higher than in the group treated with IFA. As confirmed by post-mortal analyses, gel depots were cleared from the injection site between 7 and 14 days.

Based on these promising findings, a combination therapy was evaluated (**Chapter 3**). Hereto, P407 hydrogels were co-loaded with anti-CTLA-4, anti-PD-L1 and ICG (indocyanine green). The rationale for this combination is that ICG assisted PDT therapy (based on NIR800 nm irradiation) could trigger immunogenic cell death in tumor cells, which might potentiate the effects of checkpoint blocking therapy. In vitro, ICG-PDT treated tumor MC38 cells triggered DC engulfment and maturation, confirming the premise that ICG-PDT could induce immunogenic cell death. The full hydrogel treatment regimen (PDT + anti-CTLA-4 + anti-PD-L1) with repeated PDT resulted in the strongest effects on tumor growth inhibition, especially in the CT26 model. This combination therapy furthermore induced changes in the tumor microenvironment, most remarkable was the significant increase of T-helper cells and CD8 cytotoxic T lymphocytes

(CTLs) in the tumor compared to no treatment. In the draining lymph nodes (dLNs), combination treatment tended to increase the percentage neutrophils, dendritic cells and macrophages over no treatment. These observations indicate that combined PDT and checkpoint blockade therapy with hydrogels can result in the generation of more efficient anti-tumor responses.

The research on P407 hydrogels was concluded with a chapter focusing on chemotherapy. In **Chapter 4**, 25% P407 hydrogels were loaded with the chemotherapeutic drug doxorubicin. Interestingly, the combination of P407 and doxorubicin exerted considerably more cytotoxicity on MC38 tumors in vitro as compared to freely given doxorubicin, which effect was most evident after 72 hours. A possible explanation for this observation is that a P407 micelle drug complex (~ 5 nm), as opposed to a free drug molecule, cannot be easily exported out by the P-glycoprotein on tumor cells. This mechanism renders tumor cells insensitive to chemotherapeutic drugs. As such, doxorubicin molecules cannot be exported out of the cell, which could explain the more effective tumor cell killing. The increased cytotoxicity on cancer cells, together with the slow release kinetics, could represent great benefits in addressing chemotherapy resistance as well as toxicity. In vivo imaging of injection depots showed that ICG signal in the hydrogel depot could still be detected until 7 days. When given in PBS, the ICG signals were fully cleared within 6 hours. The gradual reduction of fluorescence intensity in gel depots could indicate slower drug release in systemic circulation, and thus less side effects.

Due to the injectability, injectable gels could represent a less invasive treatment for notably tumors that are easily accessible (e.g. the skin). To access deeply residing tumors, implantable gels might be an option. In **Chapter 5**, bacterial nanocellulose (BC) was exploited as an implantable gel for optimizing the delivery of anti-CTLA-4 checkpoint blocking antibodies. BC was produced by the static cultivation of K. xylinus in 24-wells plates, which results in the production of a semi-solid gelatin like mass made up of cellulose with high water content. Various techniques – including immersion, soaking and injection – were tested to load the BC with antibodies. The injection method was the most efficient technique with limited loss of antibody. In vitro release studies showed that 50 µg of antibodies was released within 72 hours. To make the gel implantable in mice, the edges were removed with scissors, which resulted in a square-shaped product, with a length of ~ 7 mm and width of ~ 3 mm. These square shaped BC gels were then loaded with anti-CTLA-4 and implanted subcutaneously in mice. BC reduced serum antibody levels by 5 – 10 fold compared to free delivery in PBS. Subsequently, BC gels loaded with 50 µg anti-CTLA-4 were implanted in MC38 tumor bearing mice. Unexpectedly, BC based CTLA-4 blockade did not result in effective tumor growth inhibition in two independent experiments, even when the dose was elevated to 100 µg anti-CTLA-4. It is worth noting that surgery and implantation are required for the BC treatment protocol and the results might not be directly comparable with those from the P407 studies. It has been postulated that surgical stress and wound healing factors can considerably hamper immune cell function, while these factors might render tumor cells resistant to cancer immunotherapy.

This warranted an in depth literature study of the effects of surgery on cancer immunotherapy and tumor development, of which the findings are thoroughly discussed in **Chapter 6**. This review article discusses how surgical stress can suppress anti-tumor immunity. The article furthermore describes how wound healing factors may favor aggressive and even metastatic outgrowth of tumor cells. Tumor cells residing near the surgical area tend to acquire a phenotype that makes them more resistant to cancer immunotherapy. Combined immunotherapy (checkpoint blockade + therapeutic cancer vaccines) could be a promising treatment regimen to break this resistance.

2. Reflection and future perspectives

Throughout this thesis two different gel delivery systems were made and characterized. In general, the outcomes of the research were diverse and are summarized in **Table 1**:

Table 1. Advantages and considerations of P407 and BC gels

Delivery system	Advantages	Considerations
P407 hydrogel (injectable)	Quick and easy injection High loading efficiency Can be degraded Slows down antibody release and effective tumor killing Effective tumor killing when loaded with chemotherapeutic drugs	Difficult to stabilize gel formulation, premature drug release Can be hard to apply in deeper located tumors
Bacterial nanocellulose (implantable gel)	High stability and good spatiotemporal control of drug release (low serum level of drugs) Can be applied next to deeper residing tumors Easy modification of shape, pressure resistant	Requires implantation, needs removal after treatment Implant might impair organ function Surgery induces immunosuppression and outgrowth of tumor cells Depending on loading method: varying loading efficiencies

Both delivery systems have their strengths. However, the diversity of outcomes and time needed to optimize the systems also underscore the challenges of studying these gel delivery systems during pre-clinical research. The promising developments of hydrogel drug delivery systems can provide attractive solutions to mitigate common problems in cancer immunotherapy, which are often attributed to side effects and poor responses. However, to date, no hydrogel based formulations have found clinical application in cancer immunotherapy,

and the translation of results from pre-clinical drug delivery research to the clinic is still facing numerous challenges. In contrast to conventional cancer drug development, one challenge is to gain clinical approval for gel based delivery systems as their manufacturing is subjected to strict Good Manufacturing Protocol (GMP) regulations. Difficulties with upscaling the gel fabrication process, high manufacturing costs and batch/safety variations can make the development process more time consuming[1].

Besides GMP regulations, it is expected that more research is needed to explore and analyze the biochemical adjustment of gels, optimization of release kinetics, evaluation of the long term effects on health and evaluating gel treatments in additional experimental settings. Releasing immune adjuvants or checkpoint antibodies too quickly might trigger immune tolerance and induce side effects. On the other hand, a very slow release kinetic of antigens and adjuvants might lead to ineffective activation of immune cells[2]. It is still a challenge to optimize the release kinetics of hydrogels without compromising its biocompatibility and degradability. These issues were also encountered during the preparation of P407 gels. P407 gels resulted in a ~2-fold reduction in serum anti-CTLA-4 level, but the effect was considerably lower than that of IFA. A method to slow down the release kinetic is by crosslinking the polymer chains, which can prolong gel stability and drug release in vivo up for to even 40 days[3]. Studies have shown that crosslinking poloxamer gels with glutaraldehyde and chitosan can increase the gel stability, leading to prolonged drug release[4, 5]. Glutaraldehyde and chitosan were considered in this thesis. However, these compounds should be carefully considered for cancer immunotherapy. Chitosan has been reported to induce inflammation, while crosslinkers can be toxic[6]. These modifications might also reduce the degradability of the gel. Injection depots that cannot be cleared could cause inflammation and formation of sterile abscesses, conditions which should be avoided during cancer immunotherapy[7]. More research is required to address the chemical modification of the P407 in order to improve release kinetics without affecting gel degradability.

Another point of consideration is that P407 gels were kept on ice during injection to prevent premature gelation at room temperature. Injection of a cold bolus is painful and needs to be carefully considered in the clinical setting. To prevent gelation at room temperature, poloxamer 188 (P188) might be considered as additive. Adding low concentration of P188 to P407 can increase the transition (gelation) temperature of the gel to 30 °C, which prevents gelation at room temperature (19-21 °C)[8]. A higher transition temperature furthermore allows easier injection of highly concentrated P407 gels (>30%) at room temperature, which could lead to more favorable release kinetics.

In all studies, serum antibody levels were determined to assess the effects on side effect reduction. To get a better impression on side effect management, liver enzymes such as alanine aminotransferase (ALAT) or aspartate aminotransferase (ASAT) should also be measured. The concentration of these enzymes might be more indicative of the amount of the organ damage inflicted, which could further support the conclusions about the cytotoxic effects[9].

Endogenous damage protein levels might furthermore provide additional information about the (long term) effects of gel formulations on health. Primary observations (weight measurement and post-mortal skin observation) and 24 - 48 hour cell cytotoxicity studies showed that the P407 gels exerted no adverse effects. However, specific synthetic polymers have been reported to induce inflammation in the long term[10].

Throughout this thesis, the gels were administered next to subcutaneous tumors and additional research is warranted to assess whether the results are also translatable to other clinically relevant delivery methods, including intra-tumoral and intradermal delivery. Intra-tumoral treatment has received increasing attention in cancer immunotherapy research and encompasses the direct injection of compounds into the tumor. In this way, a high concentration of immunomodulatory compounds can be attained locally, which offers the advantage of activating immune cells directly in the tumor area while reducing systemic side effects[11]. There is rational to further investigate whether intra-tumoral gel based treatment with immunomodulatory compounds can result in superior treatment effects over freely given compounds. The main question is whether the extended residency time of intra-tumorally injected antibodies and adjuvants could indeed improve treatment effect without toxicity. In a study by Silva et al.[12], it was shown that intra-tumoral treatment with slow release nanoparticles delivering doxorubicin and immunomodulatory compounds resulted in significantly stronger effects on tumor growth inhibition, as compared to intra-tumoral treatment with the compounds in free solution. Intradermal delivery has also reached more interest for vaccination. Owing to the abundance of dendritic cells and lymphatic vessels, the skin is an anatomically attractive location for generating efficient immune responses with reduced immunization doses[13, 14]. It is therefore worthwhile to explore whether gel based delivery could improve intradermal immunotherapy. Considering the anatomical structures and accessibility of the skin and tumor, the choice might, however, be limited to injectable formulations for intra-tumoral and intradermal delivery.

Subcutaneous tumor models are good starting points for evaluating gel based immunotherapies, but to increase the translational relevance, the therapies should be evaluated in additional models. The injectability of P407 gels represents a great advantage as the administering procedure is less invasive. However, administering injectable gels near deeply residing tumors could be challenging. While local injection of gels might be a realistic option for tumors that grow superficially (e.g. melanomas), most patient tumors grow in organs that might disseminate to distant locations in the body, thereby challenging the applicability of injectable hydrogels. In this regard, inoculating a tumor on the opposite flank could have been considered. This experimental approach could provide insights into whether locally applied hydrogel immunotherapies can result in the generation of immunity that could eradicate distant tumors[15]. A more clinically relevant approach would be to evaluate the gel therapies in orthotopic tumor models. These tumors are grown within their usual place, which are often within the organs, and have the potential to metastasize. An alternative option would be to get access to the tumor and place a gel via implantation. Here BC gels were produced and

optimized for delivery of anti-CTLA-4 antibodies. BC gels have a high tensile strength giving them a rigid texture, which is a desired aspect of implantable gels, since the gel has to endure internal pressure exerted by the organs and internal environment of the body. The BC gels showed promising antibody release kinetics in vitro and strongly reduced serum antibody levels in mice. These results are comparable with those from earlier studies that focused on optimizing the delivery of immunotherapeutic drugs with implantable gels[16-18]. A point of consideration is that the BC could not be degraded, since animals and humans lack the enzymes to break down cellulose. Consequently, the gel needs to be surgically removed after any treatment, which can be more burdensome for the patient. Moreover, BC therapy cannot be considered for all dosing routes, including the intradermal or intra-tumoral route. Nevertheless, the impressive effects on reducing serum antibody levels could outweigh these disadvantages.

Unfortunately, implanting mice with BC loaded with anti-CTLA-4 antibodies did not result in tumor growth inhibition. This is an unexpected result, as for the P407 study, the same treatment protocol (one dose of 50 µg anti-CTLA-4) did result in effective tumor growth inhibition in the MC38 tumor model. Several explanations might underly this result. As transplantation was required, there remains a possibility that surgery and wound healing were interfering with the anti-CTLA-4 therapy, which is supported by a related study by Park et al. In this study, 4T1 breast tumor cells were inoculated in breast pads and surgically removed after tumors were established. After that, alginate gels were loaded with anti-CTLA-4 and anti-PD-1 antibodies and were implanted in the breast pads. Surprisingly, even a concentration as high as 600 µg anti-CTLA-4 + anti-PD-1 (12-fold higher than the dose tested in this thesis) failed to confer effective tumor growth inhibition. The authors speculated that surgical damage and wound healing exerted immunosuppressive effects on nearby immune cells, which might be the culprit of the resistance to checkpoint blocking therapy. These topics were further discussed in **Chapter 6**, which described how surgery tends to result in increased populations of myeloid-derived suppressor cells (MDSCs), tumor associated macrophages (TAMs) and regulatory T cells (Tregs) and elevated production of IL-10 (immunosuppressive cytokine). Increased presence and activity of these cell types have indeed been associated with resistance to checkpoint therapy[19, 20]. The same study showed, however, convincing results on tumor growth inhibition when implantable gels were co-loaded with R848 (TLR7-8 Ligand). Combinations of checkpoint inhibitor + R848 were not tested and it is tempting to speculate that treatment modalities that target different arms of the immune system could benefit post-surgical cancer immunotherapy[16]. A further interesting aspect of the study by Park et al. is that the immunotherapy was evaluated after resection of tumors. As implantation is part of the treatment protocol, there is rationale to evaluate implantable gels in combination with tumor resection. To this end, the 'partial resection model' can be a clinically relevant model for the BC mediated immunotherapy. In this model, most of the tumor mass is resected, but a small fraction of the tumor is left behind. After a certain time, the tumor regrows in the surgical area, mimicking the situation in the clinic, making this an attractive model for assessing post-surgical hydrogel based immunotherapy[21, 22].

A study by Khong et al.[23] demonstrated how this partial resection model can be exploited to evaluate post-surgical immunotherapy. ABA-HA mesothelioma tumors were injected subcutaneously, and after the tumors were established, 75% of the tumor mass was resected. The mice were treated with a combination of Imiquimod (TLR-7 agonist) + anti-CD40 afterward. The combination treatment significantly altered the post-surgical environment as reflected by increased CD4 and CD8 positive T cell infiltration in the tumor draining lymph nodes and tumor tissue. A point of consideration is that the treatments were given multiple times in free solution. High frequency treatment schemes can be burdensome for cancer patients whilst induction of side effects can complicate the clinical translation of combination therapies[24-26]. Gel delivery systems might widen the opportunities for testing more potential combinations. Sustained release kinetics might tackle the problem of complex dosing schemes and problems with side effects. The ability to release a mix of immunostimulatory adjuvants and antibodies (as well as other compounds[20]) over a prolonged period might facilitate safer and more effective prevention of post-surgical tumor relapses. Although more research is needed, BC gels could potentially represent a solution to deal with these issues as it has the potential to slowly release a high quantity of drugs over an extended period.

These topics were touched upon in **Chapter 3**, whereby P407 hydrogels were explored for combined checkpoint blockade therapy and PDT. The treatment regimen resulted in more efficient tumor growth inhibition than single therapy; the advantage was that the treatment was comprised by only a single injection of P407. This combination is worth testing for the BC implantable gel. Another option would be a combination of immunotherapy and chemotherapy (not tested here). In **Chapter 4**, it was demonstrated that P407 gels loaded with doxorubicin exerted much stronger cytotoxic effects on tumor cells over freely given doxorubicin, which effect might be attributed to the micelle-drug complexes that entered the gel. These results are in line with a study which reported that P407 micelles loaded with D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS, a P-glycoprotein inhibitor) exerted higher toxicity on cancer cells over freely given TPGS[27]. Another study showed that P407 paclitaxel therapy resulted in more efficient inhibition of melanoma growth as opposed to delivery via saline[28]. The increased cytotoxicity on cancer cells, together with the slow release kinetics, make P407 gels an attractive consideration to deal with drug resistance and side effects. It would be furthermore interesting to explore whether the higher toxicity on tumor cells leads to more synergy with other immunotherapeutic drugs, considering that doxorubicin can induce immunogenic cell death[29]. To illustrate, in a study by Zhao et al., doxorubicin was encapsulated in extracellular vesicles (EVs), which were loaded in a sprayable bio-responsive gel. The doxorubicin-EV complexes induced more immunogenic cell death than doxorubicin in free solution. The gel doxorubicin treatment furthermore strongly synergized with PD-L1 blocking therapy in preventing post-surgical tumor recurrence; the synergy was more potent compared to free doxorubicin[30].

An overall remark is that the treatments throughout this thesis were evaluated in the MC38 and CT26 tumor models, which are highly sensitive to checkpoint blockade therapy, as these tumors

bear numerous (point) mutations and express neo-antigens. To increase the translational relevance of these results, the next step is to evaluate these treatments in tumor models that tend to be more resistant to cancer immunotherapy, as many cancer patients still develop resistance to cancer immunotherapy. A candidate model would be the TC-1 lung carcinoma model, which generally reacts poorly to monotherapies. However, previous studies by Silva et al. showed that nanoparticle assisted chemo-immunotherapy and combined PDT and immunotherapy resulted in impressive effects on tumor growth inhibition of TC-1 tumors[12]. The TC-1 model can, therefore, be considered for a follow-up study to assess the P407 ICG PDT + CTLA-4 + PD-L1 checkpoint blockade therapy from **Chapter 3**. In line with this discussion, it should be stressed that many tumors require different treatment regimens. More patients are being treated with personalized medicine, indicating that customizing delivery systems with a unique release profile tailored to patient's specific tumor profile represents an important aspect of research as well in near future[31]. To address these questions, one option is to tag, for instance, near infrared (NIR) probes into hydrogels in order to follow the immune activation after hydrogel treatment in vivo[32, 33].

3. Conclusions

This thesis's research aimed to optimize cancer immunotherapy with injectable and implantable gel systems. The main focus was on controlling the release of immune checkpoint blocking antibodies. In addition, the release of photosensitizers and chemotherapeutic drugs was also studied. The injectable P407 gel was easily injectable and depots could be cleared from the injection site. Mice treated with P407 anti-CTLA-4 displayed considerably lower levels of serum anti-CTLA-4 whilst the treatment resulted in effective tumor growth inhibition. This effect was enhanced when tested in a combinatory setting with ICG (Indocyanine green) based PDT, which also induced favorable changes in the TME. P407 further slowly released the chemotherapeutic drug doxorubicin, whilst exerting strong cytotoxic effects on tumors cells in vitro. Altogether, these results indicate that P407 hydrogels can reduce the systemic drug spread and that various treatments with P407 gels resulted in effective tumor growth inhibition in vivo. Further chemical fine-tuning of P407 hydrogels is still needed, but the promising results merit further research with other exciting (combinations) of cancer therapies and other cancer models.

The BC implantable gels slowed anti-CTLA-4 antibody release in vitro and strongly reduced antibody levels in vivo. The gels did not exert side effects in vivo, had high mechanical strength but were easily adjustable in size and shape. Although BC anti-CTLA-4 therapy did not result in effective tumor growth inhibition, the results still represent good starting points to explore the role of BC as delivery system for cancer immunotherapeutic drugs. It is worth noting that the treatment condition (which involved implantation and surgery) differs from that with the P407 hydrogel. BC has only recently arrived in cancer research and additional research should further explore the experimental conditions needed for more effective BC based cancer immunotherapy. This does include the exploration of more cancer treatment modalities and

combinations of them, as combined forces hold stronger potential for more effective tumor growth inhibition.

Note

The grammar and language in this chapter was revised by *Grammarly*.

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