論 文 の 内 容 の 要 旨

論文題目　Development of Phenylboronic Acid-Based Ligand Strategies for Intratumorally Activated Sialic Acid Targeting and Drug Delivery Enhancement

（フェニルボロン酸リガンドによる腫瘍内活性化シアル酸を標的とした新規ドラッグデリバリー増強戦略の開発）

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Cells are coated with a layer of carbohydrates, *i.e.* glycans, composed of branched oligomer or polymer of monosaccharides. The family of glycans in the group that is of our interest presents sialic acid (SA) at the terminal group of the glycan chain, which regulate a range of pathological processes[1]. In healthy tissues, SA is involved in various cellular activities, such as avoiding cell-cell interaction or filtering function of kidneys, as well as acting as factors of binding sites for different pathogens and toxins. On the other hand, altered glycosylation is a trademark of almost all type of cancers regardless of the origin and stage, and SA has been reported to be involved in immune modulation and support of cancer cells in immune evasion to create a immunosuppressive microenvironment[2]. For example, SA can bind to siglec (SA binding immunoglobulin type lectin) present on the surface of T cells and exert immunosuppressive signals. The overexpression of SA has also been linked to cancer malignancy and metastasis. Cancer cells of different origins show overexpression of SA on cell surface glycoproteins and glycolipids, which contributes to the overall higher amount of SA in tumor microenvironment[3]. The ability of SA to modulate immune regulatory system is attributed as the cause for increased expression in tumor cells. This overexpression of SA is reported to be more common than oncogene markers, such as HER2/ neu, which makes SA as a universal targeting antigen for all type of tumors. Hypersialylation in cancer cells also promotes migration and apoptosis resistance, which are beneficial for tumor growth and correlated with aggressiveness and poor prognosis for cancer patients. Thus, SA represents a broad marker for tumor targeting of therapeutic agents. Nevertheless, SA is also expressed in healthy tissues, thus, hindering the development of such targeted strategies. SA can be recognized by selectins or siglecs, though they are yet to be used systematically due to their immunogenicity and lack of selectivity to tumor SA. Thus, the

development of ligands capable of recognizing tumor SA, while avoiding interaction with SA in healthy tissues, is still necessary for advancing SA targeted strategies. Boronic acids can form reversible covalent interactions with diol-containing molecules. In fact, sugars, such as the common monosaccharides (*e.g.* glucose), contain diols, and as a consequence, boronic acids have proven to be helpful molecular ligands for binding and detection, working as synthetic lectins. While the binding of boron to diol-containing molecules is commonly promoted at basic pH, the binding constant between these agents drops at neutral and acidic pHs. On the other hand, the binding of boronic acids to SA opposes this trend, and the binding constant between boronic acid compound and SA increases at acidic pHs. For example, complexes between phenylboronic acid (PBA) and sugars were shown to be stable at pH higher than their pKa, however, with pH lower than pKa only SA-PBA complexes were stable[4]. Such binding behavior of boronic acids could be exploited for selective recognition of SA at intratumoral pH conditions, which range between pH 7.2 to pH 6.5. PBA have been reported to selectively recognize SA overexpressed on cancer cells to improve the tumor targeting ability of polymeric micelles. Thus, the PBA molecules on the micelles can form ester bonds with diol-bearing molecules and shows selectivity to SA at intratumoral pH (pH 6.5). These micelles achieved enhanced intracellular drug delivery in cancer cells at intratumoral pH after attaching to the SA on the cell surfaces. Besides cancer cells, researchers have also explored the idea of using PBA as synthetic lectin to activate T cells, inducing lymphocyte proliferation, which could be applied as an IL-2 receptor stimulant[5]. As the interaction between SA and PBA is low, in this dissertation we have focused on finding PBA derivatives with higher binding affinity to SA at intratumoral pH, which can further improve the tumor targeting ability. Recently, we have reported the superior affinity of 5-boronopicolinic acid (5-BPA) for SA at intratumoral pH, which is much higher compared to PBA[6]. The unique ability of 5-BPA to have specific and high binding to SA at reduced pH makes it an excellent targeting molecule for modification of polymers and supramolecular nanocarriers, such as polymeric micelles. Thus, in my doctoral studies, I concentrated on developing targeted polymeric systems installing 5-BPA for increased binding to intratumoral SA. The first system uses 5-BPA installed polymeric micelles loading a platinum drug, *i.e.* ((diaminocyclohexane)platinum(II) (DACHPt)). The ability of these micelles for eliminating highly malignant cancer stem cells (CSCs), which are involved in tumor aggressiveness and relapse, and have been found to increase SA levels, was confirmed in tumor models of head and neck cancer. The second system focuses on lymphocyte binding polymers for promoting the differentiation and proliferation of T cells, with the goal to be applied as immunotherapies. Together, these systems are expected to elicit potent antitumor effects.

The dissertation is composed of six chapters **chapter 1** and **6** are general introduction and conclusion with future perspective.

In **chapter 2**, the prospect of 5-BPA to use as a ligand on nanoparticle for targeted cancer therapy was evaluated. First, overexpression of SA in different *in vitro* cultured murine and human cancer cell lines were verified. Relatively higher expression of SA in tumor samples compared to normal tissue collected from head and neck cancer patients was also confirmed. Thus, providing rationale to target overexpressed SA of cancer cells. The concept of pH dependent binding of 5-BPA molecule to cancer cell surface SA was proved in *in vitro* using PANC 1 cell line as it appeared to have highest amount of SA among all cancer cell lines. Verification was done by two separate studies. Firstly, the improved ability of 5-BPA to

block SA specific lectin binding at pH 6.5 was demonstrated. Then, additional experiment was performed by using rhodamine conjugated 5-BPA and directly observing higher binding of 5-BPA-rhodamine to cell surface SA which was diminished upon removal of SA through sialidase treatment. Retention of binding constant of 5-BPA after polymer conjugation was confirmed through ARS (alizarin red s) assay using 5-BPA-PEG-acetal as model polymer. Finally, superiority of 5-BPA over PBA as ligand molecule for targeting cancer cells was validated using fluorescent dye labeled 5-BPA-8-arm-PEG and PBA-8-arm-PEG. 5-BPA-8-arm-PEG demonstrated improved cellular uptake at pH 6.5 and higher tumor accumulation compared to PBA-8-arm-PEG probably by enhanced interaction with intratumor SA in acidic tumor microenvironment.

In **chapter 3**, preparation and characterization of polymers and micelles are reported. To prepare polymeric micelles poly(ethylene glycol)-*b*-poly(L-glutamic acid) (PEG-*b*-PLGA) block copolymers with narrow weight distribution and 40 units of PLGA side chain were synthesized and characterized. PBA and 5-BPA was conjugated to the end terminus of acetal-PEG-*b*-PLGA and azide-PEG-*b*-PLGA respectively. Alexa 555 & 647 dye conjugated methoxy-PEG_{10K}-*b*-PLGA were prepared to construct fluorescent micelle. Existence of boron diol on 5-BPA and PBA conjugated block-copolymer was confirmed before micelle preparation through detection of fluorescence upon mixing of boronic acid conjugated polymers with ARS. Ultimately, DACHPt loaded polymeric micelles (DACHPt/m) were constructed with 50% surface ligand (5-BPA or PBA). Micelles were characterized by measuring size, PDI, drug loading and surface charge. All the micelles size was maintained around 30 nm with narrow size distribution as previous study established micelles with larger diameter fails to achieve deep penetration inside the tumor[7]. Surface charge of the micelles were slightly negative to neutral and loading of Pt was around 30 (Pt/polymer wt/wt %). All the micelles demonstrated similar size, drug loading and surface charge thus allowing us to compare their biological activity for the ligand efficiency.

In **chapter 4**, biological activities of 5-BPA installed DACHPt loaded micelles (5-BPA-DACHPt/m) were assessed in CSC rich head and neck cancer cell line HSC2 and compared with nonligand DACHPt/m and PPA installed micelles (PBA-DACHPt/m). Before evaluating performance 5-BPA-DACHPt/m through controlled *in vitro* studies removal of SA by sialidase treatment was confirmed. Cellular uptake of the micelles was evaluated first by measuring internalized Pt in HSC2 cells using ICP MS and then tracing fluorescent-labeled 5-BPA-DACHPt/m and PBA-DACHPt/m in sialidase treated and non-treated cells using CLSM at various time point at pH 7.4 and pH 6.5. Quantification of internalized Pt revealed higher cellular uptake of 5-BPA-DACHPt/m compared to other micelles at pH 6.5. The improved cellular uptake at pH 6.5 was also analogous in CLSM study and confirmed such enhanced uptake was due to the interaction of 5-BPA and cell surface SA as uptake of ligand micelles was reduced upon cleaving of SA. Relatively lower IC_{50} value demonstrated by 5-BPA-DACHPt/m compared to DACHPt/m confirms effectiveness of ligand installation. Cellular study conducted also revealed the ability of 5-BPA-DACHPt/m to reduce SA rich CSC population. Subsequently, blood circulation and plasma clearance of the micelles confirmed comparable and long circulation profile. 5-BPA-DACHPt/m showed significantly higher tumor accumulation and retention 48 h after intravenous injection compared to other micelles in subcutaneous HSC2 tumor model. Finally, antitumor study

against orthotopic head and neck HSC2 tumor model revealed the extraordinary ability of 5-BPA-DACHPt/m to suppress tumor growth and improved survival compared to both DACHPt/m and PBA-DACHPt/m treated groups. Assessment of CSC population in tumor after treatment revealed significantly lower fraction of CSCs in 5-BPA-DACHPt/m treated animals, probably leading to better tumor suppressing ability and treatment outcome.

In **chapter 5**, we evaluated potential of polymer based synthetic lectin as immunostimulator. In an attempt to synthesize synthetic lectin PBA and 5-BPA was conjugated to 8-arm-PEG for their multiple binding site to provide multivalency. The ability of 8-arm-PEG-PBA and 8-arm-PEG-5-BPA to induce T cell proliferation was validated using immortalized human T lymphocytes (Jurkat cells) at pH 7.4 and pH 6.5. The activation ability demonstrated by the polymers were not replicated by small molecule PBA or 5-BPA verifying the necessity of multiple binding site for engaging cell surface glycan to induce proliferation similar to several binding pockets of lectin. T cells collected from mouse lymph nodes also demonstrated 3 fold higher proliferation upon incubation with 8-arm-PEG-PBA but not with free PBA. 8-arm-PEG-PBA treatment also showed significantly higher induction of CD4+ in cells collected from both lymph nodes and thymus. On the contrary, thymus cells that were treated with 8-arm-PEG-5-BPA demonstrated small but significant impact on CD8+ cells. Preliminary data from these studies suggest the potential of boronic acid conjugated polymers for *in vitro* activation of T cells and may be used as an adjuvant to immunotherapy.

5-BPA installed polymeric micelles improved tumor targeting and antitumor efficacy due to specific interaction of 5-BPA with SA at intratumoral acidic pH. Besides, the ability of our boronic acid-conjugated systems for inducing T cell proliferation suggest potential as an immunostimulator. As overexpression of SA is a universal characteristic of tumors, I expect a broad applicability of 5-BPA molecule for diagnosis and therapy of cancer, and other applications in the field of bioengineering.

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